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Developmental responses to fluctuations in environmental conditions in echinoid echinoderms

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Abstract

Animals that reside, reproduce, and develop in nearshore habitats are often exposed to strong fluctuations in abiotic conditions, including temperature and salinity. We studied the developmental responses of five echinoid echinoderms (sea urchins and sand dollars) to increased temperature and reduced salinity. We aimed to document two recently described phenomena: delay of hatching (DOH) and polyembryony. First, we found that DOH is a widespread response to reduced salinity. Hatching was delayed by 79% in *Echinarachnius parma,* 26% in *Strongylocentrotus droebachiensis,* 22% in *Lytechinus variegatus*, and 17% in *Dendraster excentricus*. Only embryos of *Arbacia punctulata* failed to delay hatching in response to reduced salinity. Second, we observed polyembryony in both of the irregular echinoids studied (*E. parma* and *D. excentricus*). In *D. excentricus*, we tested the competency of twinned and normal embryos to reach metamorphosis. We found that twin embryos generated from a single egg are both capable of reaching metamorphosis. To investigate the mechanisms underlying polyembryony, we tested whether reduced Calcium levels in low salinity seawater reduce cell-cell adhesion and allow cells to separate and develop as multiple embryos within a fertilization envelope. We also tested whether osmotic stress caused swelling of the fertilization envelope, allowing embryos more room to produce multiples, or delay hatching into a later, larger stage. However, neither reduced Calcium levels nor osmotic stress alone appears sufficient to induce polyembryony. We currently hypothesize that early suppression of the hatching enzyme plays a major role in the delaying hatching and that the swelling of the hyaline layer within the fertilization envelope may facilitate polyembryony.

Introduction

Since the Modern Synthesis, much of biological research has centered on genetics to explain evolutionary biology, marginalizing formerly important areas such as development in the process (Gilbert *et al*. 1996). While the genome, or genotype, of an organism plays a great role in guiding its biology, DNA is not the only factor that shapes an organism's physiology, behavior, appearance, growth, or development. This 'genetic instability', more commonly known as phenotypic plasticity, is the ability of a genome to produce different phenotypes under different environmental conditions (Bradshaw 2006). The study of phenotypic plasticity aims to contextualize the genome under various environmental conditions, instead of writing it off as a mere source of error or 'annoyance' (DeWitt *et al*. 1998; Gilbert 2001; Bradshaw 2006). Recently, it has been recognized that studies of phenotypic plasticity in developmental biology have challenged the Modern Synthesis in important ways and altered our approaches to the study of evolutionary and developmental biology (Gilbert 2001).

Initially the field of evolutionary developmental biology (evo-devo) applied traditional comparative embryology in order to explain the evolution of traits and the relationships among taxa (Arthur 2002). However, this subfield of developmental biology was still heavily rooted in the utilization of the genetic makeup of organisms to explain evolutionary changes and adaptations (Arthur 2002). More recently, ecological developmental biology (eco-devo) aims to further contextualize the development of organisms in the physical environments in which they evolve (Dusheck 2002). The field of eco-devo has since shown that developing organisms are capable of both interpreting and responding to environmental fluctuations, emphasizing that an organism's phenotype is not only under the control of its genotype but that the environment plays an equally large role (Gilbert 2001; Sultan 2007; Moczek *et al*. 2011). In fact,

developmental plasticity can extend the range of phenotypic variation that organisms can exhibit during early life history stages (Sultan 2007). My thesis will examine two developmentally plastic phenomena: environmentally cued hatching (ECH) and polyembryony (Warkentin 2011; Craig *et al*. 1997).

Many animal phyla develop in aquatic environments and most of these animals develop within a maternally derived structure, or egg capsule, and hatch in response to developmental and environmental cues (Warkentin 2011). Traditionally, the transition from the highly regulated internal environment of an egg capsule to the highly variable external environment has been assumed to occur when an organism reaches a fixed developmental stage (see review by Warkentin 2011). However, environmentally cued hatching (ECH) or variation in the time to hatching in response to environmental conditions, occurs across many taxa. In vertebrates, for example, eggs of red-eyed tree frogs (*Agalychnis callidryas*) have been observed to hatch from their egg capsules in response to vibrations generated from a predatory snake (Gomez-Mestre and Warkentin 2007). ECH in mammals is known as embryonic diapause, the delayed implantation of the early fetus at the blastocyst stage via either the halting of cell division, or a significant retardation of growth (Renfree and Shaw 2000; Warkentin 2011). The mammalian superfamily Pinnipedia *(*seals and sea lions) are known to undergo an obligate period of embryonic diapause, which is hypothesized to synchronize births across a population to ensure maximal offspring survival across a single habitat (Atkinson 1997). Within invertebrates the snails *Helisoma trivolvis* and *Lymnaea stagnalis* increase the synthesis and release of neurotransmitters to suppress larval development in response to chemical signals produced by crowded and starved conspecifics (Voronezhskaya *et al*. 2004). Conversely, embryos of the nudibranch (*Phestilla sibogae*), which normally hatch in 11 days, hatch in as few as 4 days in

response to a predatory crab (Strathmann 2010). These examples highlight the wide variety of hatching responses across species that provide solutions to a diverse array of potentially threatening environmental conditions.

Another potentially adaptive form of developmental plasticity is polyembryony: the production of two or more individuals from a single, sexually produced embryo (Craig *et al*. 1997). The phenomenon of polyembryony is predicted to have evolved in situations where offspring have more information regarding their environmental conditions than the mother (Craig *et al*. 1997) and seems to be especially beneficial for organisms with parasitic life stages, in which offspring are left to respond to the conditions of their host long after being deposited. For example, the parasitoid encyrtid wasp (*Copidosoma oridanum)* usually inserts one or two eggs into a moth egg, which goes through larval development relatively undisturbed by the deposited wasp egg(s). At the larval moth's final instar, the wasp egg(s) are triggered to divide, thereby producing 5-8 individuals per egg, two of which become "soldiers" that protect the host from being parasitized by another animal (Cruz 1981; Strand 1989a,b,c).

Polyembryony has also been suggested as a means of increasing clutch size when offspring numbers are limited by either maternal constraints or other factors (Craig *et al*. 1997). For example, long-nosed armadillos (*Dasypus spp*.) undergo obligate polyembryony, which is hypothesized to increase clutch size, as females only have one implantation site for the blastocyst (Galbreath 1985). Outside of the genus *Dasypus,* polyembrony is not a widely reported occurrence in mammals. The rare occurence of identical twins in humans and other mammals is considered to be "sporadic" or "accidental" polyembryony and is associated with high mortality (Craig *et al*. 1997). In general, mammals, birds, and reptiles seldom exhibit polyembryony due to the highly protective nature of the maternally derived structures in which embryos develop

(Craig *et al*. 1997).

Studies of ECH and polyembryony have further strengthened the notion that environmental cues, whether they provide information on predation, food abundance, or abiotic conditions, greatly alter development, and can induce novel phenotypes across diverse taxa. I have chosen to study these developmental phenomena in Echinoderms, which are in many ways an ideal model system for the study of such developmental phenomena because of the ease with which large numbers of embryos can be obtained and exposed to a variety of environmental conditions. Echinoderms also play an integral role in our understanding of marine ecosystem and community management. For example the keystone species concept, a fundamental principle in ecology, was first described in the ochre star (*Pisaster ochraceus)*, which has been shown to greatly influence community structure in the rocky intertidal of the western coast of the United States (Paine 1966). The life histories and reproductive modes of echinoderms are an integral part of what allows them to have an outsized influence on the biological communities in which they live, and thus my motivation for using them is derived from both ecology and developmental biology.

Echinoderms are broadcast spawners that occupy near shore habitats (Allen and Pechenik 2010). Broadcast spawning is a mode of reproduction characterized by the release of gametes into the surrounding water column where fertilization occurs, followed by embryonic and larval development (Pechenik 2010). In marine environments, broadcast spawning is associated with many benefits including: increased dispersal of gametes and larvae, freedom from desiccation stress, and the abundance of particulate organic matter available for feeding (Zeh and Smith 1985; Strathmann 1990; Levitan 2005). However, broadcast spawning prevents parental investment after gametes are released and so developing offspring must deal with environmental

stressors on their own. Environmental stressors may be particularly problematic for nearshore organisms because these habitats are characterized by a constant state of flux in temperature and salinity, which change with daily tidal variations (Cloern *et al*. 1989; Helmuth *et al*. 2002; Kaplan 2003; Allen and Pechenik 2010). Day to day salinity fluctuations due to diurnal tidal cycles are already exposing animals to conditions that are far more extreme than those predicted to occur due to climate change (National Climactic Data Center 2008). Fluctuations in temperature and salinity are widely known to affect fertilization, embryonic development, larval development, and potentially juvenile quality (Greenwood and Bennett 1981; Watts *et al*. 1981; Allen and Pechenik 2010).

Early studies in developmental biology were conducted in echinoderms (e.g. Just 1919a, b, c; 1922a, b; 1923; Harvey 1956), and their embryos continue to be used for experimentation in genomic, cellular and developmental research (e.g. The Sea Urchin Sequencing Consortium *et al*. 2006; Romano and Wray 2006; Runcie *et al*. 2012). Ernest Everett Just provided the first detailed account of the fertilization process in embryos of the Atlantic sand dollar (*E. parma*) and the effects of dilute seawater on the fertilization and early cleavage processes within this species (Just 1919a,b,c,; 1922a,b; 1923). Since the time of Just, researchers have continued to use echinoderm embryos to aid in their understanding of fundamental developmental processes and responses. For example, Romano and Wray (2006) isolated and described a novel gene in the urchin *Lytechinus variegatus*, which was found to have an integral role in the process of gastrulation. Runcie *et al*. (2012) subjected embryos of the Pacific purple urchin (*Strongylocentrotus purpuratus*) to predicted climate change temperatures and monitored gene expression for indications of thermal stress, but did not report any ECH or polyembryony in their findings (Runcie *et al*. 2012). The early developmental stages of echinoderms have therefore

been closely observed and monitored in an array of environmental settings by many researchers for nearly a century, yet these developmental responses have gone virtually unnoticed. Echinodermata is the most recent phylum in which the occurrence of ECH has been reported (Warkentin 2011). Armstrong *et al*. (2013) reported that embryos that were exposed to reduced salinity levels during early development significantly delayed their hatching. The Antarctic sea urchin (*Sterechinus neumayeri),* which relies on calcium carbonate to form its larval skeletal rods and adult skeleton, was anecdotally observed to delay hatching, as well as form smaller larvae under elevated carbon concentrations (Yu *et al*. 2013). This is particularly interesting as The Southern Ocean in which this species occurs has been dubbed as the "ocean acidification" hotspot, and is potentially affecting animals that rely on calcium carbonate to form skeletal structures. Aside from these examples, there have been no reported cases of ECH or similar responses in the development of echinoderms.

Unlike ECH, the phenomenon of polyembryony is more widely documented for the echinoderms. Most documented cases of polyembryony in this phylum have been artificially induced via either chemical exposure or through experimental embryology (Vacquier and Mazia 1968a,b; Moran and Allen 2007; Alcorn and Allen 2009). Embryos of *Dendraster excentricus, Lytechinus anamesus* and *Strongylocentrotus purpuratus* exposed to the chemical dithiothreitol (DTT) produced polyembryony in *D. excentricus*, but were lethal to embryos of *L. anamesus* and *S. purpuratus* (Vacquier and Mazia 1968a, b). Manipulations of embryos at the two or four-cell stage have been conducted since the 19th century and have been described to successfully undergo subsequent cleavage cycles (Driesch 1891;1892 as cited by Horstadius 1973). Blastomere separations conducted in the green urchin (*Strongylocentrotus droebachiensis*) and the Atlantic sand dollar (*Echinarachnius parma*) have indicated that an initial 50% reduction in

offspring size at the 2 cell stage yielded individuals that developed through the larval period normally, although development took longer than normal embryos (Alcorn and Allen 2009). Environmentally-induced polyembryony has recently been reported in the pencil urchin (*Eucidaris tribuloides*). In this species embryos were observed to produce twins, triplets, quadruplets, etc. in response to elevated temperatures and reduced salinities (Allen *et al*. accepted). Armstrong (2011) reported similar responses in embryos of *E. parma* exposed to similar thermal and salinity stressors. Aside from these reports, the earliest and only other report of polyembryony occurring in echinoid echinoderms, is in the cidaroid sea urchin *Prionocidaris baculosa*, but the conditions under which that response was elicited are unknown, and the report simply stated that this is a seemingly normal occurrence in the development of these embryos (Mortensen 1938). A better understanding of the effects of everyday stressors faced by these organisms is needed to better understand key developmental processes and to predict responses to future sources of environmental stress.

The research that I have conducted aims to study the effects of environmental stressors, namely the effects of temperature increases and/or salinity decreases, on the development of echinoid echinoderms (sea urchins and sand dollars). In my study, I subjected the embryos of six species of echinoid echinoderms to various salinity and temperature levels and followed them through early development to observe any potential differences in hatching times and occurrence of polyembryony. Studies from the Allen lab have shown that embryos of two echinoid species are capable of delaying hatching into the pluteus larval stage (Armstrong *et al*. 2013) or producing multiples (Armstrong 2011; Allen *et al*. accepted) in response to changes in the surrounding environment. The goal of my project was to determine if these phenomena are widespread in the class Echinoidea or if these observed responses are unique to only one or a few

species. Based on previous studies and preliminary experiments, I subjected embryos to ambient, or near-ambient temperatures, and reduced salinities to determine if embryos are capable of exhibiting the delay of hatching (DOH) phenomenon. To investigate the production of polyembryony, I exposed embryos of the Pacific sand dollar (*Dendraster excentricus)* to a combination of both increased temperatures and reduced salinities. I further investigated the potential ecological costs of the polyembryony response in *D. excentricus* by following individuals through the larval development period and into settlement and metamorphosis to the juvenile stage. I also tested two mechanisms by which either phenomena can come about: the first is the osmotic stress hypothesis, which predicts that the fertilization envelope of embryos incubated in reduced salinity environments swells due to the hypotonic external environment. This swelling would then allow for developing embryos to either delay hatching and continue development into a later, larger stage (such as the pluteus larval stage) or to separate into polyembryonic embryos. The second hypothesis is the reduced calcium seawater hypothesis, which predicts that embryos incubated in reduced salinity environments cannot maintain adequate cell-cell adhesion interactions in early development. The concentration of calcium ions are reduced when salinity is lowered, which is predicted to hinder cell-cell adhesion and allow cells to drift apart and continue on separate developmental paths.

Methods

Strongylocentrotus droebachiensis

Green sea urchins (*S. droebachiensis)* were ordered from the Marine Biological Laboratory (MBL) in Woods Hole, MA in February and March of 2013 and shipped overnight to Williamsburg, VA. Upon arrival animals were immediately placed into recirculating seawater tanks at 12ºC and 32 parts per thousand (ppt) until experimentation began 1-2d later. In order to induce spawning, animals were injected with 1-3 mL of a 0.5M potassium chloride (KCl) solution to induce release of gametes (Strathmann, 1987). After injection, urchins were inverted over 250 mL glass beakers containing 32 ppt artificial seawater (Instant Ocean salt mixture [Blacksburg, VA] combined with deionized [DI] water; ASW) that was chilled to 8°C. During spawning events gamete viability was assessed from different individuals by visual inspection under a compound light microscope. A small sub-sample of eggs and a drop of dilute sperm were placed on a glass microscope slide and inspected under the microscope 5-7 minutes later. The percentage of fertilized eggs was assessed by the presence or absence of the fertilization envelope (FE) in a sample of the first 50 eggs observed. Acceptable fertilization scores were 45 out of 50 eggs or more (90%+); scores lower than that were assumed to reflect poor gamete viability and additional urchins were injected until a viable pair was found. Eggs were allowed to settle to the bottom of the beaker prior to being washed by carefully pouring off excess water and re-suspending in 32 ppt ASW. After eggs once again settled to the bottom of the beaker, sperm was diluted in ASW and gently swirled to activate the cells. Immediately prior to fertilization, 2 mL of an ASW and egg mixture (1000-1500 eggs) were pipetted into 12 small Pyrex bowls containing 150 mL of chilled ASW at four salinities of 32, 30, 28, and 26 ppt, with each salinity treatment having three replicate bowls. Next, about 500 µL of dilute, activated sperm was added

to each bowl using a pipette, and gently swirled to facilitate gamete contact. Only one temperature treatment of 8°C was used in these experiments, which was maintained by placing the experimental set up in a cold room set to that temperature (Table 1).

Approximately 15 to 30 minutes after the last bowl of eggs was fertilized, fertilization score, fertilization envelope diameter (FED), and egg diameter (ED) were assessed under a compound light microscope at 100x magnification. A drop of fertilized eggs was pipetted from the bottom of each replicate bowl and placed onto a glass microscope slide. Fertilization score was assessed in the manner described above. FED and ED were measured in the first 10 eggs observed with the aid of an ocular microscope. All initial measurements were made prior to any visible signs of the first cleavage cycle. At approximately 30 hours post-fertilization (hpf), a second measurement of FED (FED2; Figure 1) was taken in the same method described above, and the first signs of hatching were observed. Hatching was sampled by gently swirling the water in the bowls to homogenize hatched and unhatched embryos, and pipetting a small sample from the middle column of the bowl. Embryos were scored by the presence (unhatched) or absence (hatched) of the FE around a spinning or swimming blastula. Hatching was sampled and measured every hour until all experimental bowls were observed to have 50 out of 50 eggs hatched.

Arbacia punctulata.

Purple urchins (*A. punctulata)* were dredged sub-tidally from the waters surrounding the Virginia Institute of Marine Sciences (VIMS) Eastern Shore laboratory in Wachapreague, VA in October of 2013. For the first day after their collection adult urchins were transferred to flow-through seawater tanks available in the VIMS labs. After the second day they were transported back to Williamsburg where they were placed into cold water (~12°C) tanks and fed *ad libitum*

until experimentation began. Experimental methods for obtaining gametes and fertilization are identical to those described for *S. droebachiensis*. Embryo cultures were maintained in a water bath set up in a large Rubbermaid container filled with water, heated with a submersible water heater, and circulated with a submersible water pump and chiller to maintain the desired water temperature of 23°C as described in Harvey 1956 .

Approximately 3 mL of washed eggs and 1 mL of dilute sperm were added to 9 Pyrex bowls containing 100 mL of ASW at three salinities of 32, 29, and 26 ppt, with each salinity treatment having three replicate bowls.

Two forms of experiments were conducted on embryos of this species; transfer experiments and delay of hatching (DOH) experiments. DOH experiments are similar to those described for *S. droebachiensis* (Table 1). For transfer experiments in this species, initial data collection after fertilization, with the exception of ED due to the profound tightness of the FE, was collected using the same methods described above. At approximately halfway through the development period (4 hpf, detailed by Harvey 1956) a visual estimate of one third of the eggs in each replicate bowl was transferred to a new bowl containing full strength ASW at 32ppt. The second third of eggs were transferred to a new bowl containing the initial salinity of that bowl, either 29 or 26 ppt (Figure 2). This produced a total of 24 experimental bowls. Thus, for each salinity treatment there was a transfer (T) and transfer control (TC) treatment, along with the initial non-transfer treatment (Figure 2). Salinities of 29 and 26 ppt had three initial non-transfer bowls, three T bowls, and three TC bowls, producing nine bowls for each salinity treatment. The only salinity treatment that was both a T and TC was the 32 ppt treatment as it is the baseline salinity and a control treatment in and of itself. Four hours later, hatching was expected to begin, and FED2 measurements were taken for all 24 bowls using the aforementioned methods, and

hatching was always sampled for the initial non-transfer bowls. Only two experiments were conducted in this manner on embryos belonging to this species, and two other experiments were conducted in January of 2014 looking at only hatching using the methods previously described for the DOH experiments for *S. droebachiensis*.

Lytechinus variegatus

Variegated urchins (*L. variegatus)* were ordered in February and March of 2013 from Carolina Biological Supply, collected from Big Pine Key, FL and shipped overnight to Williamsburg, VA. Upon arrival animals were immediately placed into warm water (22-23°C) tanks and fed until experimentation began. Experimental methods used on embryos of this species were nearly identical to those used on embryos of *S. droebachiensis* (Table 1). Only DOH experiments were conducted on embryos of this species, and similar data were collected from experimentation conducted on embryos of *S. droebachiensis*. The only notable difference is the incubation temperature in which embryos were maintained. Since this species is generally a warm water species, residing off the Florida shores, embryos were exposed to temperatures ranging from 21-24°C, otherwise, embryos were exposed to identical salinity treatments and sampling conditions.

Eucidaris tribuloides

Pencil urchins (*E. tribuloides*) were ordered in October and December of 2012 from Carolina Biological Supply, collected from Big Pine Key, FL, and shipped overnight to Williamsburg, VA. In addition, in October of 2014, one shipment of urchins was ordered from Gulf Specimen Marine Laboratories in Panacea, FL and shipped overnight to Williamsburg, VA. Upon arrival animals were immediately placed into warm water (22-23°C) tanks and fed until experimentation began. DOH experiments were conducted using methods identical to those used

on *A. punctulata*. The salinity treatments used were identical; however, the incubation temperature ranged from 24-26°C, which was determined from experiments conducted by Schroeder (1981). All data collected for DOH experiments are identical to the aforementioned data collected from other species. Additionally, transfer experiments were conducted on embryos of this species. Methods used for these experiments are comparable to those detailed for *A. punctulata*. The only difference is the incubation temperature for this species which ranged from 24 to 26°C (Table 1).

Finally, an experiment was conducted on embryos which appeared as if they were in the process of twinning (pseudo-twins) in October of 2014. Gametes were obtained from adults and fertilized using methods previously detailed for other species. Fertilized eggs were incubated in a 24-26°C water bath, in 9 Pyrex bowls, containing 100 mL of 32, 29, or 26 ppt ASW, and allowed to develop for approximately 4-5 hours. After this period, unhatched normally developed, twinned, pseudo-twinned class 2, and class 3 embryos (Figure 3) were sorted via mouth pipette and individually maintained in 96-well plates. Approximately 24 to 48 hpf, sorted larvae were scored if they were present, and if they appeared normal, to determine if pseudo-twinned embryos can "recover" from the pseudo-twin phenotype and develop into morphologically normal larvae.

Echinarachnius parma

Eastern sand dollars (*E. parma*) were ordered in September of 2012 and 2013 from Gulf of Maine Inc., collected subtidally from Cobscook Bay, ME and shipped overnight to Williamsburg, VA. Upon arrival, animals were transferred to cold water (~12°C) tanks for holding until experimentation. Adults were spawned, gametes collected and fertilized using the aforementioned methods. Transfer experiments, using methods similar to those described for *A.*

punctulata, were conducted on embryos of this species. The only notable difference was that the TC replicates were not utilized for these experiments for this species. Additionally, DOH experiments, identical to those described for other species, were also conducted, and similar ED, FED, and FED2 data collected. The incubation temperature used for experimentation on embryos of this species hovered between 12 and 13°C (Table 1).

Dendraster excentricus

Western sand dollars (*D. excentricus*) were hand collected from the sand dollar beds between the months of April and August of 2014 in East Sound on Orcas Island, WA (Figure 4). Animals were transported back to Friday Harbor Laboratories (FHL) and placed in large, outdoor flow-through seawater tanks with a heavy layer of sand coating the bottom of the tanks. Animals resided in those tanks and were picked at random for experiments, after which they were placed in another outdoor tank designated for spawned animals to prevent animals from accidentally being re-used in future experiments.

The first experiment conducted on embryos of this species was a fertilization assay. Adults were spawned, gametes collected, and fertilized using methods identical to those already described. Eggs were distributed in 2 mL aliquots using a micropipette and gently releasing them into the experimental bowls to avoid shear stress. Sperm were then distributed into bowls containing eggs in 100 µL aliquots, also utilizing a micropipette, and then gently stirring each bowl to facilitate fertilization. Seven salinity treatments were used for the fertilization assay, ranging from 32 ppt, and decreasing in increments of two, to 18 ppt. Each salinity treatment had three replicate bowls, and all bowls were left out on the lab bench to develop in room temperature conditions (~19-22°C). Fertilization was scored out of a sample of the first 50 eggs observed, and cleavage was monitored and scored up to the fourth cleavage (16-cell) cycle. The

fertilization assay was utilized to determine at what salinity embryos of *D. excentricus* fail to reach 50 % fertilization. The experimental salinities determined from this experiment were 32, 29, and 26 ppt.

Subsequent experiments aimed to assess the variation in the frequency of either DOH or polyembryony in embryos exposed to different environmental conditions. Experiments were set up using the 32, 29, and 26 ppt salinity treatments, with three replicate bowls for each salinity, and using two temperature treatments. One treatment is termed "heated" and set up in a water bath ranging from 19-23°C, and the second treatment is termed "ambient" and set up in the flow-through sea table in which the water temperature ranged from 12-15°C depending on the day (Figure 5). The percent fertilization was collected immediately after the addition of sperm for both temperature treatments, and after approximately 5-7 hpf bowls were checked for signs of polyembryony. The development of embryos in each bowl was scored as being normal, twins, triplets, quadruplets, more (> 4 embryos within a single fertilization envelope), pseudo-polyembryonic (Figure 3), or abnormal (see Figure 6 for images of categories used for the scoring of embryos). Similar to my methods for measuring fertilization, the first 50 embryos observed were the ones scored. Twinned and normally developed embryos were isolated via mouth pipette from experimental trial runs in which polyembryony was observed to occur frequently. Isolated embryos were transferred to petri dishes and placed on a pedestal - to prevent water from flow-through seawater tanks entering and flooding the replicate bowls - for approximately 24 hours during which they were exposed to ambient seawater temperatures. The following day, embryos were observed under the dissecting microscope to check for early signs of larval development and swimming. Twinned and normal embryos that appeared healthy and alive were transferred to 250 mL beakers. Larval beakers were maintained in a flow-through

seawater tank and under a stirring rack and fed every other day a mixture of approximately 250 cells/mL from each *Dunalliela tertiolecta* (green algae), *Isochrysis galbana* (yellow algae), and *Rhodomonas* sp. (red algae) cultures (Sthrathmann 1987; algal concentrations modified from Schiopu *et al*. 2006). Algal densities were counted from each culture using a haemocytometer after which, appropriate volumes of algae were centrifuged for 3-5 minutes at 12,000 RCF, and the supernatant was pipetted off prior to the algal pellets being re-suspended in the larval cultures. Larvae were maintained through to the settlement period. Water changes were conducted every other day by siphoning 100-150 mL of the water in which the larvae were developing and filtering it through a 30 μM mesh to prevent the accidental loss of any larvae. In addition, beaker position in the stirring rack was altered every other day to control for any effects of placement. A second rearing protocol was utilized for sets of twins that were isolated and maintained together to determine the likelihood of a pair of twins to reach settlement and metamorphosis into the juvenile stage. In this case, a set of twins were isolated and maintained in small 2 mL cuvette tubes (Figure 7) and placed into a rack, which was then placed into the flow-through seawater tank. For these rearing experiments, water changes were conducted by manually transferring the larvae via mouth pipette into a new cuvette containing fresh Millipore filtered seawater (FSW) however, larvae were still fed the same concentration of 250 cells/mL of each algal type. Water changes and feedings were still conducted every other day.

Additionally, DOH experiments were also conducted on embryos of *D. excentricus* at both heated and ambient temperature treatments (Table 1). Data collection for these experiments was nearly identical to those collected for other species, with the addition of polyembryony (detailed above) and cleavage data also being collected. Cleavage to the 2, 4, and 8+ cell stage (Figure 8) was scored 1, 1.5, 2, and 3 hpf in the heated water treatment and 1, 2, 3, and 4 hpf in

the ambient water treatment. In a subset of the experiments conducted, Calcium reduced seawater (Ca^{2+}RSW) was used as an additional salinity treatment to help elucidate the mechanisms underlying either DOH or polyembryony. Calcium- free seawater was made using a recipe detailed in Strathmann 1987, and modified by first calculating the proportion of seawater to DI water needed to make 26 ppt seawater, and then substituting DI water for Calcium-free seawater to create Ca^{2+}RSW. When used, Ca^{2+}RSW, was treated exactly the same as the standard salinity treatments of 32, 29, and 26 ppt and the exact same data were collected from this salinity treatment as the other treatments (Figure 5).

Transfer experiments conducted on embryos of *D. excentricus* in which embryos were fertilized only 32 and 26 ppt salinity treatments were utilized, and maintained at room temperature conditions (~19-23°C). Transfers were conducted at time intervals of 0, 15, 60, 90, and 240 minutes post-fertilization (Figure 9). Three reservoir bowls were used for each salinity treatments, each containing equal aliquots of 2-3 mL of the eggs obtained from the female parental pair obtained for that experiment. Eggs were fertilized in those reservoir bowls and then immediately transferred via mouth pipette to bowls of the other salinity (T), and to another bowl at the same salinity (TC) for the 0 minute time point. For the subsequent transfer time points, embryos were always transferred by mouth pipette from the reservoir bowls and into the T or TC bowls. After 5-6 hpf all bowls were scored for polyembryony using the methods detailed above for the polyembryony experiments.

The final experiment conducted on embryos of *D. excentricus* is a single "mass spawning" experiment with the goal of generating as many twinned embryos as possible for subsequent isolation and larval rearing. Only 29 and 26 ppt salinity treatments were used for this experiment, and only the heated water bath, and lab bench temperature treatments were used for

this experiment. Adults were spawned, and gametes collected, fertilized, and distributed according to the previously described methods. In the morning, 4 unique female/male pairs were used, embryos from 2 pairs were placed in the heated water bath (~20°C), and embryos from the remaining two parental pairs were placed in bowls left on the lab bench to develop in room temperature conditions (~19-23°C). Only fertilization score data were collected immediately post-fertilization. Approximately 5-6 hpf, polyembryony was scored in all of the experimental bowls using methods detailed above. Afterwards, more sand dollars were spawned, and an additional 3 female/male pairs were obtained from that afternoon spawning. Similarly, embryos from 2 female/male pairs were incubated in a 20°C heated water bath, and the embryos from the remaining parental pair were left to develop on the lab bench in room temperature conditions. Fertilization scores and polyembryony data were collected from these female/male pairs in the same time frames and using the same methods.

Finally, pseudopolyembryony experiments were conducted on embryos of *D. excentricus* using identical methods to those detailed for *E. tribuloides*. These experiments were conducted to determine if pseudo-polyembryonic embryos of *D. excentricus* can continue to develop into larvae that appear normal.

Statistical analyses

All statistical analyses were conducted using SPSS version 22. All DOH data were analyzed using a binary logistic regression analysis in which salinity, age (hours post fertilization – hpf) and their interactions were coded as covariates, and hatching status (hatched or unhatched) was the dependent variable. Data collected from *S. droebachiensis, A. punctulata, L. variegatus,* and *E. parma* were analyzed separately in this manner, with a unique analysis for each trial. For DOH data collected from *D. excentricus,* temperature was included as an additional covariate, as

were its interactions with salinity and age. To test for replicate bowl effects, we ran univariate analyses of variance (ANOVAs) and found no noticeable difference in the results obtained from those analyses in comparison to the results obtained from the binomial regressions. I have reported only the results for DOH data from the binomial regression analyses as they are more appropriate for dichotomous dependent variables and continuous independent variables than are the ANOVAs.

ED and FED data obtained from replicate bowls were averaged, and analyzed using a general linear mixed model. The analysis of ED data for *S. droebachiensis, A. punctulata, L. variegatus, E. tribuloides,* and *E. parma*, were carried out with trial coded as a random factor, and salinity coded as a fixed factor. FED data were analyzed similarly to ED data for the same species, with the addition of time as another fixed factor, along with its interaction with salinity. ED and FED data analysis for *D. excentricus* were analyzed similarly with the addition of temperature as another fixed factor in the analysis along with its interactions with salinity and/or time. Post-hoc, Bonferroni corrected, pairwise comparisons were conducted to determine if there were any differences between salinity treatments. Because normality of the data is an assumption of general linear mixed models (and ANOVAs in general), residuals were calculated, plotted, and tested using the Kolmogorov-Smirnov and the Shapiro-Wilk tests to check for fit of the data to a normal distribution. Data reported should be assumed to be insignificant $p > 0.05$ for both normality tests unless stated otherwise. Data that significantly deviated from both tests were transformed in attempts at fitting the data to a normal distribution. In situations in which interactions yielded $p > 0.250$ they were excluded, and the model was re-run (Quinn and Keoug 2002). In these cases, p-values from the reduced models with excluded interactions (modified models) are the p-values that are reported.

Results

Delay of Hatching

Strongylocentrotus droebachiensis

Binomial logistic regression analysis for each trial found that salinity, age (hours post-fertilization; hpf), and their interaction all had a significant ($p < 0.001$) effect on hatching (Table 2). On average, embryos of *S. droebachiensis* exhibited a 25 % delay of hatching in salinities of 26 ppt relative to controls at 32 ppt (Figure 10). Embryos exposed to intermediate salinities of 30 and 28 ppt showed intermediary delays in hatching in comparison to embryos exposed to 26 ppt seawater (Figure 11A-C). All embryos were observed to hatch as blastulae, with very few showing very early signs of gastrulation.

We hypothesized that a potential swelling of the ED or FED under reduced salinity conditions would provide increased volume for the embryo to delay hatching and continue to develop to a later larger stage of development. To determine how salinity affects ED and/or FED, I compiled data from three replicate parental pairs (Figures 12A-C) and analyzed the parental pair means (Figure 13) using ANOVA. Salinity had a significant effect on egg diameter (Table 3), with eggs exposed to 26 ppt salinities increasing in diameter by over 7 μm relative to 32 ppt controls. Assuming the egg is spherical in shape, this equates to a 4% increase in diameter, and 15% increase in egg volume (Figure 13). Post-hoc, Bonferroni corrected pairwise comparisons revealed that all salinities were significantly different ($p < 0.028$) from one another, except for 30 ppt and 32 ppt ($p > 0.900$). Salinity significantly affected FED ($p < 0.001$) but time ($p = 0.343$) and the interaction between salinity and time did not ($p = 0.811$; Table 3). Post-hoc, Bonferroni corrected pairwise comparisons for FED revealed that 26 ppt and 28 ppt treatments were not significantly different from one another ($p = 0.458$), nor were 30 ppt and 32 ppt

treatments ($p > 0.900$), but that the two groupings (26/28 vs 30/32) are significantly different from one another ($p < 0.003$; Figure 3). Embryos maintained at 32 ppt seawater showed a slight 2 μm shrinkage between FED1 and FED2 measurements (Figure 3).

Arbacia punctulata

Embryos of *A. punctulata* exhibited no overall delay in hatching into at salinity 26 ppt (Figure 10). Salinity did prove to have a significant effect on hatching in two of the three trials (Table 4). However, when plotted, mean hatching proportions of embryos incubated in salinities of 29 and 26 ppt did not show a great difference from embryos exposed to full strength seawater (Figure 14A-C). In the first experimental trial, eggs incubated at 26 ppt fertilized and developed at very low numbers, and so overall hatching for that salinity treatment for that experiment was omitted from (Figure 10). For the second experimental trial, salinity and age were both found to have significant effects on hatching (Table 4). Analysis of hatching data for the third experimental trial yielded results that indicate that salinity and age are not significant predictors of hatching (Table 4). Upon closer inspection of the data generated from the third trial, we noticed that for the first time point no hatching was observed, and that for the third and final time point all of the embryos had hatched (Figure 14C). Therefore all of the variation in hatching is from the second time point; this drastic pattern in the data interfered with the capabilities of the binary logistic regression to adequately interpret the data. Artificial manipulations of the data from the third experimental trial, where a few embryos were changed from being unhatched to hatched in the first time point, yielded results that were comparable to those from the first two trials. All embryos, from all experimental trials, were observed to hatch as blastulae.

ED and FED data were compiled from three replicate parental pairs (Figures 15A-C) and their mean responses analyzed using ANOVA (Figure 16). Salinity had a significant effect on

egg diameter (Table 5), but eggs exposed to 26 ppt salinities increased in diameter by only 1 μm, about a 1 % increase in diameter, and 3 % increase in egg volume. Post-hoc, Bonferroni corrected pairwise comparisons indicated that 26 ppt and 29 ppt treatments did not significantly differ from one another with regards to ED ($p > 0.900$) however, 26 ppt and 32 ppt treatments differed significantly from one another ($p = 0.019$), while 29 ppt and 32 ppt treatments did not ($p = 0.084$). FED1 and FED2 differed slightly across salinities, across the three parental pairs reported (Figure 15A-C; Figure 16). Post-hoc, Bonferroni corrected pairwise comparisons showed that the 26 ppt treatment significantly differed from both the 29 ppt and 32 ppt treatments ($p < 0.001$), but 29 ppt did not differ significantly from 32 ppt ($p > 0.900$). In the first experimental trial, ED and FED1 data were identical as the FE did not raise appreciably above the surface of the egg. Salinity and temperature were shown to have a significant effect on envelope diameter, however their interaction did not have a significant effect (Table 5).

Lytechinus variegatus

Embryos of *L. variegatus* were repeatedly observed to fail to undergo normal development at salinities of 26 ppt, and so data for that salinity treatment were omitted from the overall DOH, ED, and FED analyses as well as the three replicate trial figures (Figure 10; Figures 17-19). Embryos incubated in salinities of 28 ppt were observed to delay by 22 % when compared to the hatching of embryos incubated at full strength seawater (Figure 10). However, for the second experimental trial, embryos failed to undergo normal development in quantities adequate for hourly scoring at salinities of 28 ppt and so data from that salinity treatment for that trial are omitted as well. For the remaining treatments, age, salinity, and their interaction were shown to have a significant effect on hatching (Table 6). All embryos were observed to hatch as blastulae.

ED and FED data were compiled from three replicate parental pairs (Figures 18A-C) and plotted together (Figure 19) for an ANOVA. Salinity had a significant effect on egg diameter (Table 7), with eggs exposed to 26 ppt salinities increasing in diameter by almost 8 μm, which is about a 6 % increase in diameter, and a 21.6 % increase in egg volume. Post-hoc, Bonferroni corrected pairwise comparisons for salinity showed that most salinity treatments were significantly different from one another ($p < 0.001$), except for the 32 ppt and 30 ppt were not found to be different from one another ($p = 0.096$). Plotted FED1 and FED2 showed differences across salinities, across the three parental pairs reported, especially with regards to the first and third trials (Figure 18A-C). However, a mixed model ANOVA showed that salinity did not have a significant effect on FED, whereas time did have a significant effect, and the interaction between the two variables was also not significant (Table 7). Bonferroni post-hoc comparisons showed that none of the salinity treatments were significantly different from one another ($p > 0.765$). Embryos showed a 2-3 μm swelling between FED1 and FED2 measurements (Figure 19).

Eucidaris tribuloides

Only one DOH experiment was conducted on embryos of *E. tribuloides*. This experiment was a pilot experiment in which I took hatching measurements at wide time intervals. Embryos exposed to 26 ppt were observed to have remained within their FE at 16 hours post fertilization and were observed to have reached close to 50 % hatching 10 hours later (Figure 20). It is unclear that whether or not the observed responses were genuine DOH, or if the offspring generated from this particular parental pair were especially sensitive to the experimental treatment and responded abnormally. While the data is not sufficient to clearly indicate a delay,

there seems to be a hint of a DOH-like response that would require further, more detailed experimentation.

ED did not differ markedly across salinity treatments (Table 8). However, embryos exposed to 26 ppt seawater are reported to have increased ED by about 3.5 μm, which equates to a 3.7 % increase in diameter, and an 11 % increase in egg volume overall (Figure 21). Post-hoc, Bonferroni corrected pairwise comparisons show that salinity treatments are not significantly different from one another ($p > 0.352$). Salinity and temperature separately were found to have a significant effect on FED however, the interaction between the two variables did not have a significant effect on FED (Table 8). The 26 ppt treatment was observed to have embryos in which FED1 measurements that are about 3.3 μm larger than the other two treatments (Figure 21). A slight trend indicating an increase in FED2 was observed across salinities. Embryos maintained at salinities of 29 ppt have FED2 that are about 1.3 μm larger than embryos maintained at full strength seawater. Embryos kept at 26 ppt have FED2 measurements that are about 1.5 and 2.83 larger than the 29 and 32 ppt treatments respectively (Figure 21). Bonferroni post-hoc comparisons for FED indicate that 29 ppt treatment was not significantly different from either the 26 or 32 ppt salinity treatments ($p > 0.127$), but 26 ppt and 32 ppt salinity treatments were found to be significantly different from one another ($p = 0.024$). These data were obtained and analyzed from a single female-male pairing, and so further experimentation is needed to definitively validate these results.

Echinarachnius parma

Hatching data was gathered from a single transfer experiment conducted on embryos of *E. parma*. Only data from the non-transfer treatments was analyzed using a binomial logistic regression and a univariate ANOVA in which salinity was a fixed effect and replicate bowls

were coded as the random effect. For embryos of this species, salinity was found to have a significant effect on hatching (Binary logistic regression; Wald's $X^2_1 = 96.253$; $p < 0.001$; $e^{\beta} = 0.001$). When a univarite ANOVA was run, salinity was still found to have a significant effect on hatching, whereas replicate bowls were not (Table 9). This data was collected at only one time point, (11.5-14.5 hpf), embryos were transferred about half-way through the developmental cycle (about 7 hpf) into full strength seawater. Transfer seems to slightly increase the likelihood of hatching; embryos first fertilized at 29 ppt and 26 ppt salinities and then transferred to 32 ppt seawater both experienced a 0.08 increase in hatching proportion in comparison to embryos maintained at the initial fertilization salinities (Figure 22).

Salinity was found to have a significant effect on ED (Table 10), with eggs exposed to 26 ppt increasing in diameter by over 34 μm, a 29 % increase in diameter, and an overall 118 % increase in egg volume (Figure 23). This is by far the most drastic increase in egg volume reported in all of the species studied, and a key assumption of this relatively crude calculation is that eggs of this species are uniformly spherical in shape. However, based on the equally drastic results observed by Armstrong *et al*. (2013) for hatching (Figure 10), this observed difference does not seem to be out of the realm of possibility. With regards to FED1 and FED2, salinity, time, and their interaction were all found to have significant effects on FED (Table 10). Embryos maintained at 26 ppt experienced an overall 36 μm and 20 μm increase in FED1 and FED2 respectively when compared to embryos incubated at 32 ppt for the entirety of the developmental period (Figure 23). Bonferonni post-hoc comparisons were conducted on salinity for both ED and FED data and all salinities were reported to be significantly different from one another ($p < 0.021$).

Dendraster excentricus

An initial fertilization assay was conducted on gametes of *D. excentricus* to determine how salinity affects fertilization (Figure 24). My data show that 50% of eggs fertilize at 26-28 ppt (Figure 24). For salinity trials, I selected 26 ppt as representative of a low salinity at which development is still viable.

I used a binomial logistic regression to analyze hatching data in *D. excentricus* with age, salinity and temperature as predictor variables, along with their interactions. I used the 'sometimes-pool' method for reporting p-valuesof various interactions between and among variables (Quinn and Keough 2002, pg. 260). The full model, with all possible interactions, was run first. Interactions that yielded p-values> 0.250 were then excluded, and the reduced model was run. I report the p-values generated from the full model and the reduced models in Table 11 however, p-values from the reduced models are the only ones that I discuss here. Trial 3 was not re-run as none of the interactions yielded a $p \geq 0.250$. I hypothesize this to be due to the lack of variance in hatching proportions observed in trial 3 of the ambient water temperature treatment (Figure 25C) in comparison to the first two ambient water trials (Figures 25A-B) or all three heated water trials (Figures 27A-C). The results of figures 25A-C and 27A-C are summarized in Table 12. Overall, it was found that temperature, age, salinity, and their interactions had a significant effect on hatching across the three replicate parental pairs, except for in the cases of temperature for trial 2 and the interaction between age and salinity in trial 3 (Table 12). For the two replicate parental pairs in which temperature was found to have a significant effect on hatching, embryos maintained at cooler temperatures took longer to reach 100% hatching in comparison to embryos cultured in heated treatments. Salinity, also affected the hatching of embryos, with embryos cultured in 26 ppt seawater taking longer to hatch than embryos

maintained in 32 ppt salinity seawater (Table12). Overall, embryos of *D. excentricus* exhibited a17% delay in hatching relative to controls when maintained at 26 ppt seawater and ambient water temperatures (Figure 10). DOH data for embryos cultured at heated water temperatures were not included in the overall species comparison graph, because DOH data obtained from other species were collected at or near ambient water temperatures for each species.

ED and FED data were compiled from three replicate parental pairs at both temperature treatments (Figures 26A-C and 28A-C) and grouped together (Figure 29) for an ANOVA. Salinity ($p < 0.001$) but not temperature ($p = 0.310$) had a significant effect on ED (Table 14) with eggs maintained in 26 ppt seawater at ambient and heated water temperatures exhibiting a 7μm increase in ED, which equates to a 5 % increase in ED (Figure 29A-B) and a 1.9 % increase in egg volume. Post-hoc, Bonferroni-corrected pairwise comparisons indicate that all three salinity treatments are significantly different from one another ($p < 0.001$). In contrast to the ED data, neither salinity nor temperature had a significant effect on FED (Figure 29; Table 14). When normality tests were conducted on FED data, both the Kolmogorov-Smirnov and Shapiro-Wilk tests were significant (Table 13), indicating a lack of fit of the data to a normal distribution – an assumption of ANOVA. Therefore, FED data for *D. excentricus* were log-transformed using the $\log_{10}$ function, which yielded non-significant differences between the transformed data and a normal distribution, according to both the Kolmogorov-Smirnov and Shapiro-Wilk tests (Table 13). After the data transformation, temperature, salinity, time, and their interactions did not significantly affect FED. When interactions that yielded $p > 0.250$ were removed from the model, salinity and temperature still did not have a significant effect on FED however, time did have a significant effect ($p < 0.001$; Table 14).

For embryos of *D. excentricus,* FED2 data were only collected in treatments that produced polyembryonic embryos in order to more directly test for the potential effect of FED swelling or shrinking on the production of this developmental response. Table 15 summarizes FED2 data in treatments and replicates which produced polyembryonic embryos. In all three of the heated water experimental trials, embryos exposed to reduced salinities produced polyembryonic embryos. We hypothesized that polyembryonic embryos would have larger FEDs, either as a result of the extra space required by the presence of multiple embryos, or as an underlying cause of polyembryony. However this was not the case. In three of the six cases in which polyembryony was observed in the experiments summarized in Table 15, a negative difference was observed between polyembryonic FED2 and non-polyembryonic FED2, indicating shrinkage of FED2 rather than swelling of FED2 in polyembryonic embryos (Table 15). In the cases in which swelling was observed (a positive difference between polyembryonic FED2 and non-polyembryonic FED2), the range of increase (1.1667 to 5.7667) was less than the range of decrease (-0.2833 to -10.01667).

To determine whether salinity delays early development, embryos of *D. excentricus* were scored for cleavage in the first few hours after fertilization. Three experimental trials were conducted (Figures 30-31 and 33-34) and the means of two developmental stages plotted (Figures 32A-B and 35A-B) for ambient and heated water treatments. Data obtained from those experiments failed to fit a normal distribution even after several transformations, and so a Kruskal-Wallis non-parametric test was used for the analysis of these data. Trial, temperature and salinity did not significantly affect development to the two-cell stage or the four-cell stage and beyond (Table 16). Time was the only variable that had a significant effect on cleavage; as

time increases the likelihood of reaching the two or four cell stage increases (Table 16), a common characteristic of all development.

Cross-Species DOH, ED, and FED Comparisons

Hatching data were analyzed across species, in which the percent increase in time to hatching across salinity treatments was calculated relative to the control salinity of 32 ppt (Figure 10). Data for *E. parma* were taken from Armstrong *et al*. (2013) and the remaining data come from my own work. Both species identity and salinity treatment significantly affected the percent change in time to hatching (Figure 10; Table 17; 2-way ANOVA; $F_{4,14} = 35.133$; $p < 0.001$ and $F_{3,14} = 3.719$; $p = 0.037$ respectively) but there was no significant interaction between the two (2-way ANOVA, $F_{3,14} = 1.551$; $p = 0.242$). Bonferroni-corrected post-hoc comparisons revealed that the increased time to hatching for *E. parma* was significantly greater than for all other species ($p < 0.001$) and that values for *A. punctulata* were significantly lower than for all other species ($p < 0.050$).

Egg diameter and fertilization envelope diameter varied greatly across species as well (Figure 36). All species, except for *E. tribuloides* were shown to have EDs that were significantly affected by salinity. Of those that varied in ED with salinity, embryos of *E. parma* exhibited the strongest effect (Figure 36A). With regards to FED, all species except for *L. variegatus* and *D. excentricus* were shown to have significant salinity effects on FED. Again, embryos of *E. parma* exhibited the most obvious of responses to salinity in comparison to all of the other species studied (Figure 36B).

Polyembryony

Polyembryony data were analyzed across three replicate *D. excentricus* parental pairs in which embryos were scored as either being normal, polyembryonic, pseudo-polyembryonic, or

abnormal (failure to cleave, irregular cell division etc.) development (Figure 6). An ANOVA showed that salinity, temperature, and their interaction significantly affected the percentage of normal, polyembryonic, and pseudo-polyembryonic development (Figure 37A-C; Tables 18A-C) but there was no significant interaction of either treatment or their interaction on abnormal development (Figure 37D; Table 18D). Post-hoc, Bonferroni corrected comparisons showed a significant difference between heated and ambient temperatures ($p = 0.003$), across all salinities ($p < 0.008$) except the 29 ppt and 32 ppt salinity treatments which were not significantly different from one another ($p = 0.826$).

Across 13 parental pairs, the frequency of polyembryony ranged from 0% to 48.53% (Figure 38A). A 2-way ANOVA in which temperature and salinity were coded as fixed effects, and parental pair as a random effect, showed that the two experimental treatments each had a significant effect on percent polyembryony, but their interaction did not (Table 19). Bonferroni post-hoc comparisons show no significant differences between salinity treatments of 26 ppt to 29 ppt, and 29 ppt to 32 ppt ($p = 0.186$ and $p = 0.965$ respectively), but comparing 26 ppt to 32 ppt revealed a significant salinity effect ($p = 0.032$).

Across the same 13 parental pairs, the frequency of pseudo-polyembryony ranged from 0% to 68% (Figure 38B). A 2-way ANOVA in which temperature and salinity were coded as fixed effects and parental pair was coded as a random effect, showed that the two experimental treatments had a significant effect on the frequency of polyembryony, but their interaction did not (Table 19). Because the interaction between temperature and salinity was highly insignificant ($p = 0.868$), it was omitted and the test was run again and yielded the results presented in Table 19. Bonferroni post-hoc comparisons show significant differences between salinities of 26 ppt to 29 ppt, and 29 ppt to 32 ppt were not significantly different from one another ($p = 0.362$ and $p =$

0.188 respectively), but a comparison of 26 ppt to 32 ppt salinity treatments was significant ($p = 0.008$).

I hypothesized that a reduction in free calcium ions present in 26 ppt seawater was a potential mechanism by which polyembryony can occur. Embryos fertilized and incubated in reduced calcium seawater in the early developmental stages did not exhibit any noticeable differences in proportions of polyembryonic or pseudo-polyembryonic development, regardless of temperature treatment (Figure 39).

To determine at what developmental stage polyembryony is determined, embryos of *D. excentricus* were fertilized in 32 ppt seawater and 26 ppt seawater, and transferred into the alternate salinity at four different time intervals that are roughly representative of key cleavage and/developmental stages. Three experimental trials were conducted using three unique female/male pairs (Figures 40-41), and were compiled into two plots (Figure 42A-B): one for polyembryonic development and the other for pseudo-polyembryonic development. For these three parental pairs, incidences of polyembryonic development was generally low, reaching an average maximum of about 10 % in embryos that were control transferred from 26 to 26 ppt seawater 15 minutes after fertilization (Figure 42A). However, incidences of pseudo-polyembryonic development exhibited interesting trends that are worth noting, although, no statistical analyses were conducted on data generated from these experiments. Embryos that were transferred from 26 to 26 ppt at 240 minutes post-fertilization (mpf) exhibited a mean of 19.09% pseudo-polyembryonic development (Figure 42B). When compared to embryos that were transferred from 26 to 32 ppt at the same time point, pseudo-polyembryony dropped to 9.56% (Figure 42B). This is a 10% decrease in the frequency of this developmental response across sibling embryos, which were maintained under similar conditions throughout most of the

developmental period except for the final two to three hours prior to hatching. When these values are compared to embryos first fertilized in 32 ppt seawater and then transferred to 32 ppt and 26 ppt, percent pseudo-polyembryony only reaches an average of 0.89 % and 3.33 % respectively (Figure 42B). When compared to embryos that were never transferred from 26 ppt and 32 ppt seawater, percent pseudo-polyembryonic development is 16.54 % and 0.22 % respectively (Figure 42B). I found this to be especially interesting because I had assumed that pseudo-polyembryonic individuals were destined for death, but these values indicate that embryos are potentially capable of responding to their environment and changing their embryonic morphology relatively late in the developmental cycle.

To follow up on the observations obtained from the transfer experiments (Figures 40-42) preliminary experiments were conducted on pseudo-twin embryos of *D. excentrictus* (Figure 43; Table 20A) and *E. tribuloides* (Table 20B). I observed a high proportion of isolated class 2 and 3 pseudo-twin embryos (see Figure 3 for schematic of classes) continuing through development to produce larvae that appeared normal (Tables 20A-B). For *D. excentricus,* class 4 embryos (twins) are reported to continue through to the larval stage in proportions that are almost three times less than those reported for the other classes (Table 20A). For *E. tribuloides*, I exclusively sorted class 2 and 3 pseudo-twin embryos and over 90 % of embryos from both classes were observed to reach the larval stage successfully (Table 20B). These observations indicate that pseudo-twins, regardless of the degree of the embryos' ingressions, are capable of reorganizing their cellular structure to form pluteus larvae that appear to be morphologically normal.

Field Data and Observations

Temperature spikes during Fraser River episodes (Figure 44) are reported to reach a maximum of 15.5°C, however those data are reported from an underwater monitoring station housed at the Friday Harbor research laboratories (red dot Figure 4), located about 21 kilometers away. During these same events, salinities have been reported to reach a minimum of 21.3 ppt, well below the 26 ppt minimum used in experimental trials. Field data collected en route to and at Orcas Island, San Juan, WA, USA indicate that sand dollars experience temperature and salinity fluctuations even during lulls in Fraser River episodes (Tables 21A-B; red arrows Figure 44). All data was collected at high tide. Temperature data taken at the sand dollar bed indicate that animals are experiencing conditions that are similar to laboratory conditions during the daylight hours. On 25 July 2014, temperatures at East Sound reached an average of 19.6°C and an average salinity of 31.8 ppt (Table 21B). The stressful temperatures used in experimental trials averaged about 20°C, identical to temperatures observed in the field. Based upon the data obtained from field observations made at East Sound, Orcas Island we expect temperature increases and salinity decreases in shallow surface waters to frequently be even more drastic than those reported from the underwater monitoring station.

Discussion

My study aimed to explore the potential impacts of changing environmental conditions on development in echinoid echinoderms that occupy environments that are rapidly changing. Echinoids are also especially relevant study systems because they are model organisms for the fields of developmental, genetic, and molecular biology (Just 1919a, b, c; Just 1922 a,b; Just 1923; Romano 2006; Sea Urchin Genome Sequencing Consortium 2006). My research has shown that in response to environmentally relevant fluctuations in temperature and salinity echinoids exhibit unusual developmental responses such as changing the time to hatching, and the number of individuals produced from a single zygote. Environmental induction of changes in early development may carry-over to later developmental stages (Jarrett and Pechenik 1997; Pechenik *et al*. 1998; Qian and Pechenik 1998; Pechenik *et al*. 2003), which could influence individual fitness, and potentially affect population or species level processes.

Delay of Hatching

Hatching in echinoid echinoderms occurs through two methods: biochemical degradation of the fertilization envelope (FE) by the hatching enzyme (HE) and mechanical breakdown of the FE by the beating of cilia (Mozingo *et al*. 1993; Gilbert 2000). Echinoid echinoderms typically hatch as blastulae (Staver and Strathmann 2002) and continue to develop in the water column to the pluteus larval stage, after which most species feed to fuel development to metamorphosis and the juvenile stage (Pechenik 2010). A shared characteristic of newly hatched echinoderm embryos is the ability to swim (Staver and Strathmann 2002). Swimming is speculated to have evolved as a means of providing planktonic embryos and larvae with a method to escape predators (Straver and Strathmann 2002). Because predation rates on planktonic embryos and larvae have been reported to be as high as 100% (Vaughn and Allen 2010), swimming may

confer adaptive advantages to newly hatched larvae and the inhibition of swimming could potentially increase larval susceptibility to predation. Delay of hatching (DOH) in echinoid echinoderms is associated with a delay in swimming and can inhibit the larval ability to ingest particulate food in the surrounding water and potentially have great repercussions on offspring survival.

In the six species studied, salinity was consistently seen to cause changes in time to hatching. Environmentally cued hatching (ECH) has been reported extensively in the literature, and has been reported across many phyla (Warkentin 2011). My study is the first of its kind to document the relatively widespread nature of this developmental phenomenon within the phylum Echinodermata. Two recent studies have reported delays in hatching in embryos of *E. parma* and *S. neumayeri* in response to reduced salinity and elevated carbon concentrations respectively (Armstrong *et al*. 2013; Yu *et al*. 2013). Roller and Stickle (1985) have shown salinity-induced delays and temperature-induced increases in time to hatching in different embryonic and larval developmental stages in three echinoid echinoderm species: *S. droebachiensis, S. pallidus, S. purpuratus,* and *P. ochraceus*. Compared to this previous research however, my study provides much more detail into the time taken to hatching in various species. Furthermore, my study examined various echinoids from wide-ranging locations extending from the warm, tropical waters of Florida to the cold waters of the Pacific Northwest and the Atlantic Northeast. The geographic disparity across the species studied has provided great insight into the potential widespread nature of this recently documented phenomenon in Echinodermata.

Multiple experiments conducted on a variety of species revealed several responses to reduced salinity. My results revealed a spectrum of developmental responses to salinity alterations. In the most extreme of reactions, embryos of *L. variegatus* were observed to

consistently fail to develop properly in reduced salinity conditions. On the more moderate end of the spectrum, embryos of *S. droebachiensis* increasesd time to hatching by up to 25%, but still hatched as blastulae or early gastrulae. In the mildest of reactions *A. punctulata* experienced a significant, but minimal delay of hatching in reduced salinity conditions (Figure 10). Embryos of *D. excentricus*, an irregular echinoid (sand dollar), were observed to have an intermediate increase in time to hatching (about 17 %) at reduced salinities when compared to embryos of other species. I was unable to conduct experiments of the same detail on embryos of *E. tribuloides* as obtaining viable gametes from reproductive adults proved to be quite difficult. Preliminary observations suggest the potential for salinity-induced increases in the time to hatching in embryos of *E. tribuloides*. This initial observation is promising for follow-up experimentation, and comparison to responses of embryos of *E. parma* exposed to similar stressors. I predict irregular echinoids (e.g. *E. parma*) and cidaroid urchins (e.g. *E. tribuloides)* to be more developmentally similar in their responses to environmental fluctuations than regular echinoids (e.g. *S. droebachiensis, A. punctulata, L. variegatus). E. tribuloides* belong to the order *Cidaroida,* thought to be the closest living ancestors to modern regular urchins. and are considered to be primitive (Schroeder 1981; Bennett *et al*. 2012). Variations in development across the more closely related regular urchins, *S. droebachiensis*, *L. variegatus*, and *A. punctulata,* and irregular urchins, *E. parma* and *D. excentricus,* strongly suggest that phylogenetic relationships do not dictate hatching responses within the echinoids. Instead, DOH may be a unique characteristic of development that greatly varies to different extremes on a species or population level.

It is not clear whether or not a delay of hatching in response to reduced salinity conditions is adaptive. ECH is generally shown to be an adaptive response to changing

conditions (Atkinson 1997; Voronezhskaya *et al*. 2004; Strathmann 2010; Warkentin 2011). Embryos can alter their hatching responses to various cues, such as parasite or predator presence, or in response to changes in environmental conditions. Wedekind (2002) showed that embryos of whitefish (*Coregonus* sp.) hatch early in response to water-based cues generated from conspecifics infected with a virulent egg parasite. This phenotype is associated with parasite evasion and is thought to aid in reducing the risk of contracting the disease (Wedekind 2002) however, costs of premature hatching and implications for the continued development and survival of offspring were not discussed. Similarly, red-eyed tree frogs (*Agalychnis callidryas*) have been shown to hatch about one-third of a developmental cycle early in response to predation by social wasps (*Polybia rejecta;* Warkentin 2000*)*. This behavioral phenotype is attributed as being an immediately effective defense to wasp predation, however, older frog hatchlings are known to incur improved survival against aquatic predators (Warkentin 2000). This early hatching behavior in red-eyed tree frogs, while immediately beneficial, is assumed to carry costs for survival in the larval (tadpole) life history stage (Warkentin 2000). Due to the widespread literature highlighting the adaptability of ECH, I predict that hatching delays observed in the species studied are potentially adaptive. Remaining within the fertilization envelope in response to unfavorable conditions may allow for the developing organism to reach a stage at which it can better handle the external environment. This can be thought of as similar to embryos of anamniotic eggs delaying hatching and increasing the incubation period until aquatic conditions return (rainfall for amphibians and high tide for fishes) to flood developed embryos, and trigger hatching (Martin 1999). I initially hypothesized that a swelling of the FE, caused by relatively reduced external ion concentrations that accompany reduced salinity seawater, would allow the developing embryo more room to grow to a later, larger stage while still retaining the

protective benefits of the FE. Salinity was found to have a significant effect on the FE diameters (FED) in embryos of most species except for *D. excentricus* and *L. variegatus*. This statistically non-significant salinity effect on FED in embryos of *D. excentricus* is possibly due to the differences in the methods used for collection of these data for this species in comparison to data collected from other species. Embryos of *L. variegatus* were observed to be more sensitive to salinity reductions than the other species, and the lack of salinity effect on FED in this species can be a general product of low salinity tolerance. Regardless of these salinity induced FED responses, visual examination of plotted FED data across species (Figure 36B) suggests that FED swelling alone is not an adequate mechanistic hypothesis in the production of DOH responses.

None of the embryos I observed exhibited a delay as visible as that reported by Armstrong *et al*. (2013) in *E. parma*. Embryos of *E. parma* were reported to have delayed up to 32 hpf and embryos were observed to continue development into the pluteus larval stage while still within the FE (Armstrong *et al*. 2013). Embryos of the species I have studied were all observed to have hatched as blastulae or early gastrulae. Although the delays I report are not as extreme, I hypothesize that modifications at the molecular level may have occurred that could potentially produce the phenotypes reported by Armstrong *et al*. (2013) and me. Such changes include a delay in the genetic expression of the gene encoding for the hatching enzyme or inhibition of ciliary beating that assists with the mechanical degradation of the FE.

The hatching enzyme (HE) is thought to be one of the first proteins coded by the zygotic genome based on the lack of HE transcripts detected in unfertilized eggs (LePage and Gache 1990; Ghiglione *et al*. 1997). HE has been compared to mammalian collagenases in that both classes of enzymes degrade peptide bonds found in the extracellular matrix of cells/bacteria and the embryo respectively (LePage and Gache 1990; Mozingo 1993). Expression of HE is

characterized as being transient in that transcripts accumulate during cleavage cycles and disappear at hatching (LePage and Gache 1990).

Due to the early activation of HE, I hypothesize that eggs experiencing reduced salinity conditions at fertilization, or during very early cleavage cycles, become determined in their delayed or non-delayed fate. This hypothesis is supported by the negligible increases in hatching ~6% in *E. parma* embryos fertilized at reduced salinities and transferred to full strength seawater half-way through development. In fact, the mechanical act of transfer seems to have a greater effect on hatching proportions as control embryos fertilized at full strength seawater and transferred into full strength seawater decreased hatching by 12%. Additional observations of cleavage to the 2-cell stage, 4-cell stage, and beyond in embryos of *D. excentricus* indicate that hatching enzyme expression, rather than cleavage delay, is the main cause of delayed hatching. Delays in early cleavage are negligible compared to the 3 hour delay in hatching observed in embryos maintained in 26 ppt seawater. Based on these findings, I believe that because cleavage is not being delayed, early suppression or delay in HE expression is the ultimate cause for DOH. Observations made from the transfer experiment in *E. parma* and the monitoring of early cleavage across salinities in *D. excentricus*, indicate that hatching enzyme expression is determined and expressed or suppressed nearly immediately after the sperm meets the egg.

Hatching is also achieved through the mechanical degradation of the FE by the beating of cilia located on the periphery of a hatching blastula. I have not looked at the effects of salinity on ciliary beat in any of the species studied, however, research conducted on marine polycheate worms has shown that hypotonic (reduced salinity) conditions can elicit inhibition of (ciliary) activity, that gradually wears off as the animal acclimates (Wells and Ledingham 1940). Inhibitions of ciliary action in response to reduced salinity conditions in marine worms aligns

well with the general trends of delayed hatching I observed across species. Embryos across species were observed to hatch at the blastula stage, with very few showing signs of more progressed development while retaining the FE. These observations suggest at a temporary shock-type inhibitory response to reduced salinity conditions, in which embryos exposed to a salinity reduction are temporarily inhibited in utilizing their hatching mechanisms.

Delayed hatching may carry more costs for the organism than it does benefits. Animals that delay hatching and remain within their fertilization envelope longer, may have to make up for lost feeding time by remaining in the plankton longer to acquire the nutrients necessary to fuel metamorphosis and juvenile settlement. This increased time feeding may make larvae more susceptible to predation and can potentially increase larval mortality (Vaughn and Allen 2010). Furthermore, in one of the replicate trials (trial 3) conducted in Armstrong *et al*. (2010) embryos exposed to reduced salinities were never observed to hatch, even past 80hpf, and instead were reported to have died within their envelopes. In the case of offspring generated from that unique parental pair, hatching mechanisms seem to have totally been inhibited thus resulting in increased offspring death rather than increased survival in harsh conditions.

Polyembryony

This study reports the first case of naturally induced polyembryony in embryos of *D. excentricus*, and the third report of the natural induction of polyembryony within echinoid echinoderms (Armstrong 2011; Allen *et al*. accepted). Past studies have exposed embryos of *D. excentricus* to dithiothreitol (DTT) chemical treatments and have observed polyembryony (Vacquier and Mazia 1968a). I exposed embryos of *D. excentricus* to both increased temperatures (~20°C) and decreased salinities (26ppt) and found that the combination of both treatments increase the likelihood of polyembryonic responses. Conversely, normal

development was reported to decrease under identical experimental exposures. The magnitude of this decrease in normal development, 52 %, is quite shocking as this could have great implications for the survival, fecundity, and overall fitness of members of this population. Statistical analyses conducted on these data have shown that temperature, salinity, and their interaction have significant effects on normal, poly, and pseudo-polyembryonic development, but that these three variables do not have a significant effect on abnormal development. Embryonic development is not necessarily being halted or impaired by the experimental manipulations, which suggests that the embryos are capable to cope with their relatively stressful environments. Armstrong (2011) and Allen *et al*. (accepted) have both shown in embryos of *E. parma* and *E. tribuloides* respectively, that a combination of decreased salinities and increased temperatures elicit similar polyembryonic responses.

The study of the impacts of multiple stressors affecting the physiology and development of organisms is a surging field in biology (McBryan *et al*. 2013). Various studies have shown that animals respond differently and more profoundly to exposure from multiple stressors than exposure to single stressors. Vasquez *et al*. (2013) found that independently reduced salinities and increased temperatures had minimal effect on the development of newly fertilized horseshoe crab eggs (*Limulus polyphemus*), but that the combination of the two stressors had a lethal effect. Yu *et al*. (2011) showed that elevated CO_2 and decreased pH exposure did not inhibit larval development of the Pacific purple urchin (*S. purpuratus*), but larvae were often smaller after exposure to both stressors. Observations of polyembryony, failed development, and smaller larvae in these marine taxa were elicited through exposure to environmentally relevant changes in abiotic conditions, highlighting the importance of studying the synergistic effects of

biologically relevant changes in the conditions that these animals experience (Armstrong 2011; Allen *et al*. accepted; Yu *et al*. 2011; and Vasquez *et al*. 2013).

Twinned embryos that were maintained through the larval period (Figure 45A), and a pair of twins that were maintained to settlement (Figure 45B) suggest that polyembryonic embryos are capable of continuing to later developmental stages. Artificial manipulations of embryos at the two-cell stage have shown that simulated twinning has yielded viable larvae and metamorphosed juveniles that take longer to develop and are smaller at settlement (Moran and Allen 2007; Alcorn and Allen 2009). My observations align well with studies of artificially twinned and reared embryos (Moran and Allen 2007; Alcorn and Allen 2009), and imply that later stages of development are not halted as a consequence of multiple production. Larvae, however, may still incur costs such an increased need to remain in the plankton in order to acquire necessary nutrients for metamorphosis. Similar to delayed individuals, this prolonged planktonic period might increase the risk of predation (Vaughn and Allen 2010). Polyembryony is slightly different in that there are multiple individuals with identical genetic makeup and so the costs of predation can theoretically be offset.

The underlying mechanisms of polyembryony are also unclear in the literature. I originally hypothesized that a disruption in cell-cell adhesion due to reduced salinity conditions might be the causative agent. Cadherins, calcium dependent adhesion proteins, are cell-surface proteins that maintain pathways of communication and adhesion between cells (Lodish *et al*. 2000). Reduced salinity seawater, which has reduced free calcium ions (Ca^{2+}), might limit adhesion potential in cells at the two-cell stage, causing them to fall apart and continue on two separate developmental paths. Embryos cultured in Ca^{2+}RSW were not shown to produce multiples in high frequencies. Embryos maintained in Ca^{2+}RSW and heated water temperatures

exhibited higher rates of abnormal development, but when compared to full-strength seawater, the frequencies did not seem to be out of the ordinary. I now believe that a combination of ion reductions, and not Ca^{2+} reductions alone are potentially responsible for the production of this response.

Due to the inconclusive results gained from embryos exposed to Ca^{2+}RSW, I now believe that the hyaline layer has a role in causing polyembryony. The hyaline layer is a secondary extracellular membrane that surrounds the developing embryo and is implicated in maintaining cells' proximity to one another throughout cleavage cycles (Citkowitz 1971; McClay and Fink 1982). I now hypothesize that the lack of this membrane in irregular echinoids, such as *D. excentricus* and *E. parma* (Citkowitz 1971), allows for the production of multiples. Additionally, embryos of *E. tribuloides,* considered to be the closest living ancestors to regular urchins, have also been reported to lack a hyaline layer (Schroeder 1981). McClay and Fink (1982) have discussed that, in the early development of regular echinoids such as *L. variegatus, S. droebachiensis,* and *A. punctulata,* cell-hyaline interactions were shown to be relatively stronger than cell-cell interactions. The strength of this interaction weakens as development progresses, and cells-cell interactions become much stronger (McClay and Fink 1982). The absence of the hyaline layer and its known integral role in maintaining cell-cell adhesion seems to be a promising explanation for the production of multiples in the species in which this phenomenon has been shown (*E. parma, E. tribuloides,* and *D. excentricus).*

Several transfer experiments were conducted in attempts to better explain the stage at which polyembryony is determined in embryos of *D. excentricus*. Unlike the HE there are no reports in the literature of a gene or gene product that can have a role in the production of this phenomenon. Interestingly the results from the transfer experiments indicated an unexpected

degree of plasticity in the production of pseudo-polyembryonic development. Throughout the research period pseudo-polyembryonic individuals were virtually ignored because they were not the primary focus of the study. Embryos that were fertilized at 26 ppt and transferred to 32 ppt seawater at 240 minutes post-fertilization showed an average decrease of roughly 10 % pseudo-polyembryonic development when compared to embryos that were control transferred from 26 ppt to 26 ppt seawater. This suggests that embryos are capable of changing their developmental morphology even as late as 4 hpf, which is roughly 2 hours prior to when hatching begins in embryos maintained at warm temperatures. These results are similar to observations made about the timing and capability of reorganization of sea star embryos whose fertilization envelope was stripped early in development using a urea treatment (Allen personal observation). Seastar embryos exposed to urea exhibited loss of cellular organization and made a "mat" of cells on the bottom of the well plates in which they were cultured, however a few days later, morphologically normal bipinnaria larvae were observed in place of the mats of cells (Allen personal observation). The shift in pseudo-polyembryonic embryos to normal embryos of *D. excentricus* even late in development suggests some sort of shift in cellular organization on the part of pseudo-polyembryonic embryos that is plastic in response to fluctuations in environmental conditions. However, this assertion needs to be investigated in further detail, especially because, when embryos were transferred from 32 ppt seawater to 26 ppt at 240 minutes post-fertilization, % pseudo-polyembryony remained quite low (only a 2.44 % increase with transfer to decreased salinities). This discrepancy in percent pseudo-polyembryony across different transfer treatments indicates that embryos initially exposed to favorable conditions become fixed in their response whereas embryos initially exposed to less favorable conditions maintain some level of plasticity.

The waters of the San Juan Islands have intense tidal flows and are well-mixed (Engie and Klinger 2007). These characteristics of flow and circulation of water in the San Juan Archipelago were modeled using drift cards that were released at different locations and retrieved in diverse and distant areas (Klinger and Ebbesmeyer 2001). The characteristics of flow in combination with regular intrusions of freshwater from the Fraser River have great implications for the [benthic] fauna of the region. Data collected in the field validated experimental conditions used in the laboratory in the study of polyembryony. Water temperatures at the sand dollar beds in East Sound, Orcas Island, San Juan, WA, USA reached temperatures of about 20°C, which closely resembled experimental temperature treatments. Salinity conditions at an underwater monitoring station at the Friday Harbor labs reached minimums (21 ppt) that were well below the salinity treatments used in laboratory conditions (26 ppt). Therefore the conditions to which I subjected embryos are within the range of values they experience in nature.

Nearshore marine environments may be commonly affected by terrestrial sources of freshwater input. For example, the Great Barrier Reef (GBR) in Australia receives freshwater input from the Burdekin River that can affect flushing of the GBR lagoon and drastically change salinity conditions experience by organisms living there (Wolanski and Jones 1981). Low freshwater input to the Chesapeake Bay in the year 2002 was shown to be associated with increased water quality while extremely high levels of freshwater input in 2003 corresponded with a decrease in the water quality of the Bay (Acker 2005). These significant differences in annual water quality are intimately tied to excess levels of nutrient loading which can trigger phytoplankton blooms that can have damaging effects across the entire bay ecosystem (Acker 2005). Given that nearshore organisms may frequently encounter fluctuating temperature and

salinity conditions, it is conceivable that they have experienced strong selection to cope with these environmental changes. In my study, it is important to note that polyembryony is highly variable across *D. excentricus* parental pairs. I have observed about 80 % multiple production in one replicate bowl, and in another experimental trial observed 0 % multiple production under identical conditions. This indicates that there is a great variation within this population with regards to embryonic propensity to produce multiples. Whether this trait is heritable or not is unknown. However if propensity of polyembryony is heritable then evolutionary forces could alter the abundance of this trait in the population causing the trait to increase or decrease in abundance depending on the increase or decrease in fitness this trait might confer. Studying the heritability and underlying mechanisms controlling these responses would greatly further our understanding of the potentially adaptive benefits of these traits.

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Figures

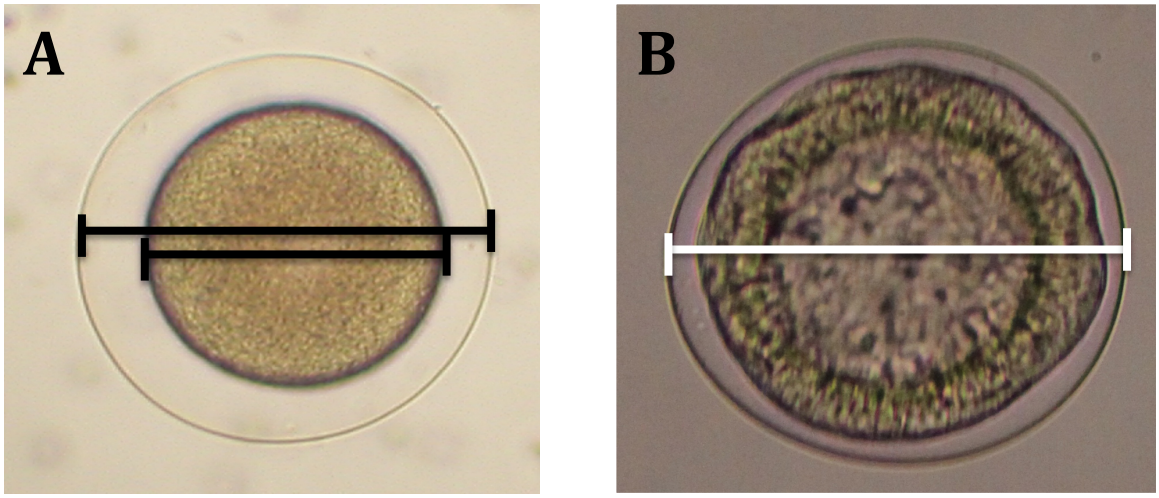


Figure 1: Image of a recently fertilized egg (A). Inner line indicates how egg diameter (ED) was measured, and the outer line indicates how fertilization envelope diameter (FED1) was measured immediately after fertilization. Image of a fully developed blastula (B), shortly before hatching. Line indicates how FED2 was measured prior to hatching.

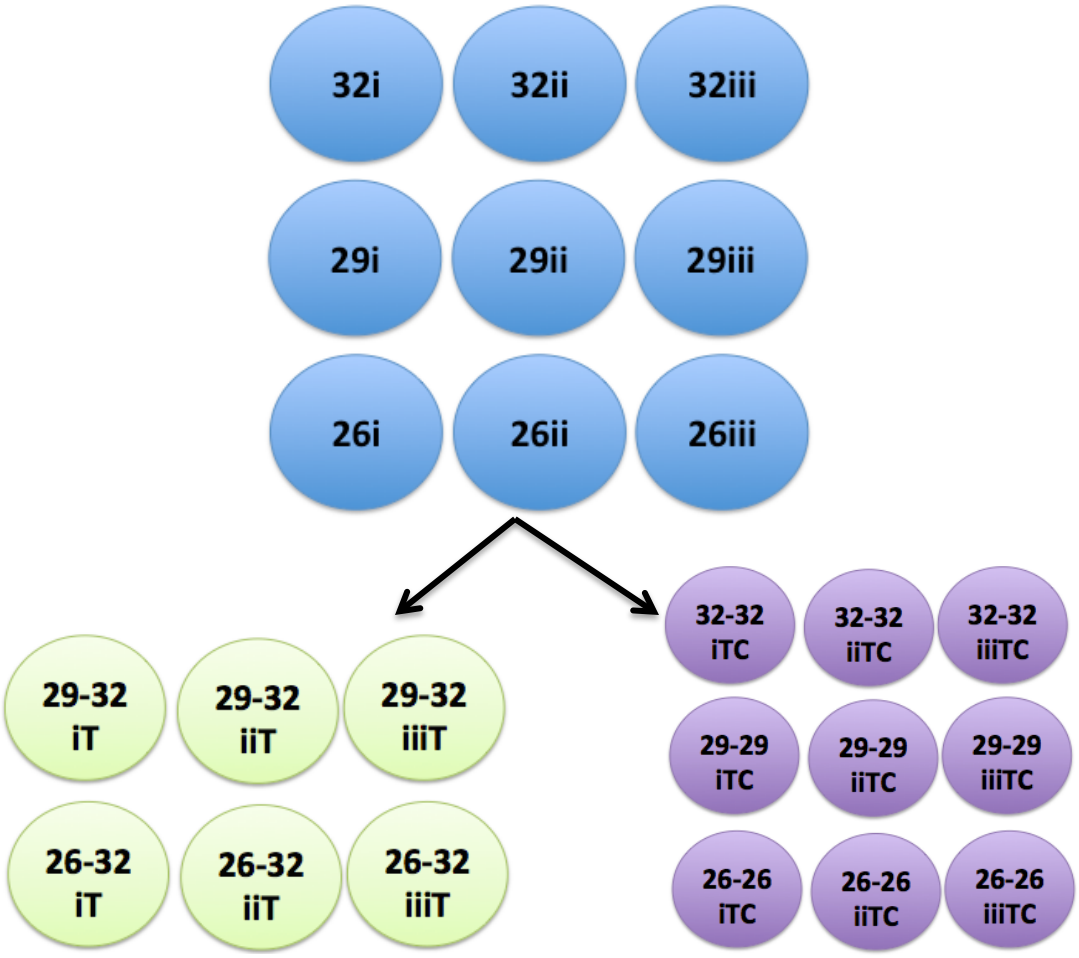
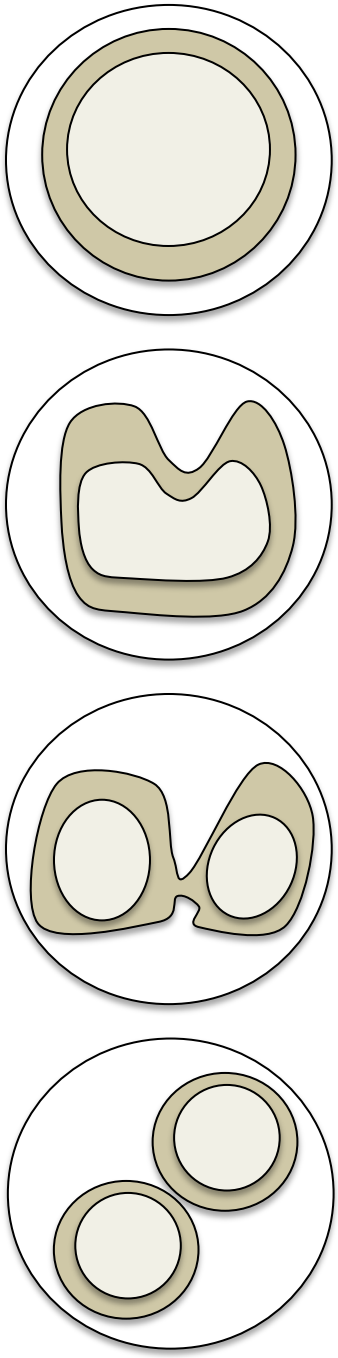


Figure 2: Schematic depicting the experimental design of transfer experiments conducted on *A. punctulata, E. tribuloides,* and *E. parma* (with the exception being that TC treatments were not employed for *E. parma*). Blue circles indicate replicate bowls that contained embryos that were initially fertilized at the stated salinities, purple circles indicate control embryos transferred to the same salinity in which they were fertilized (TC), and green bowls indicate embryos which were transferred from the initial stressful initial salinity treatment to full-strength seawater (T). Transfers were done half-way through the developmental cycles of each species, which was approximately 4, 4-5, and 7 hours post-fertilization for embryos of *A. punctulata, E. tribuloides,* and *E. parma,* respectively.



Class 1: Normal development; 0 % ingression of the blastula, or no discernible sign of an ingression is observed.

Class 2: Pseudo-twin that is characterized as having a slight, <50 %, ingression of the blastula.

Class 3: Pseudo-twin that is characterized as having a major, >50 %, ingression of the blastula, but has not been completely severed.

Class 4: Fully divided twins that had an ingression which completely severed the two blastulae.

Figure 3: Schematic of the four different development classes of pseudo-twins. Class 1, defined as being normal development, appears as a single blastula with no/unnoticeable ingression in the embryo. Class 4, is the other end of the spectrum, and is defined as full twinned blastulae, appearing to be completely separated. Class 2, is classified as an embryo that has a noticeable but less than 50 % ingression. Class 3 is classified as an embryo that has an ingression that is greater than 50 %, but the embryo has not split into two distinct blastulae. Abnormal development is classified as an embryo appearing irregular in its development and is regarded as having failed developmentally. Only embryos that were observed to still have a fertilization envelope surrounding the embryo were scored and then sorted.

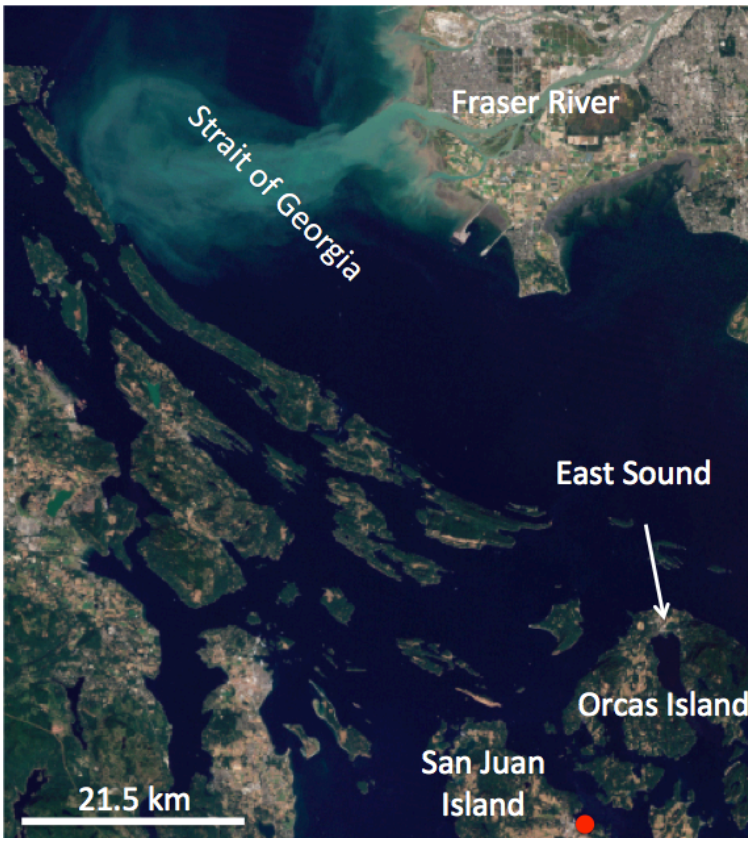


Figure 4: Image illustrating the flow of freshwater as indicated by the light blue plume dispersing into the Strait of Georgia. Freshwater percolates down into the waters of the San Juan Islands before heading out to the open ocean. Adult sand dollars were collected from East Sound (indicated by white arrow). Sand dollar beds are susceptible to fluctuations in temperature and salinity caused by freshwater input from the Fraser River.

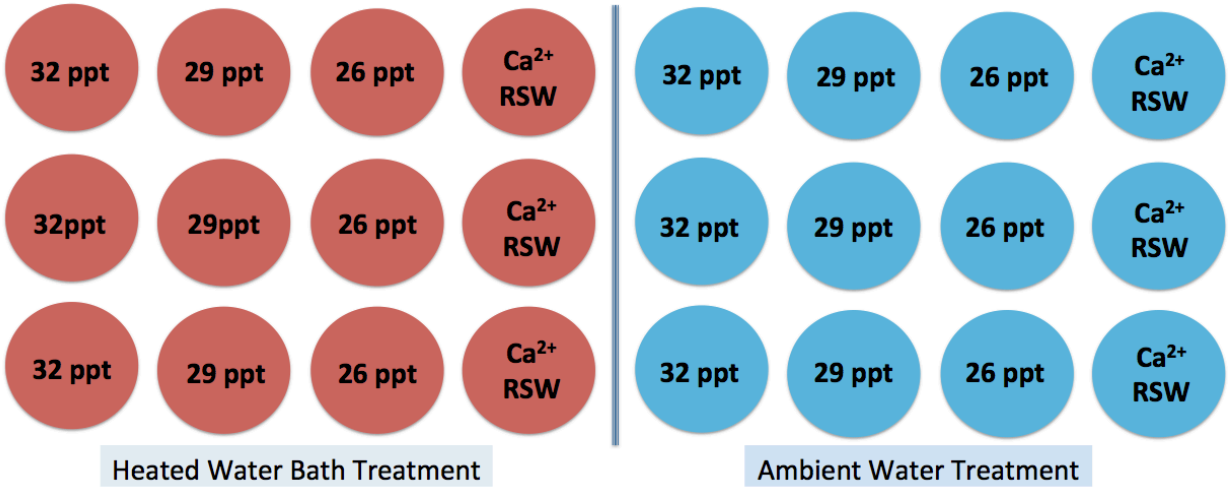


Figure 5: Schematic depicting the salinity and temperature treatment replicates in which embryos of *D. excentricus* were exposed. Heated water bath treatments ranged from 19-23°C and ambient water treatments ranged from 12-15°C. This experimental set up is nearly identical to that used for all DOH experiments in other species. The only notable differences are the salinity treatments for *S. droebachiensis* and *L. variegatus* being 32, 30, 28, and 26 ppt. DOH experiments conducted for all other species were done only using one temperature treatment, which is ambient, for each species, as indicated in the methods. Calcium reduced seawater (Ca^{2+} RSW) treatment was used only on embryos of *D. excentricus,* and only in a subset of trials.

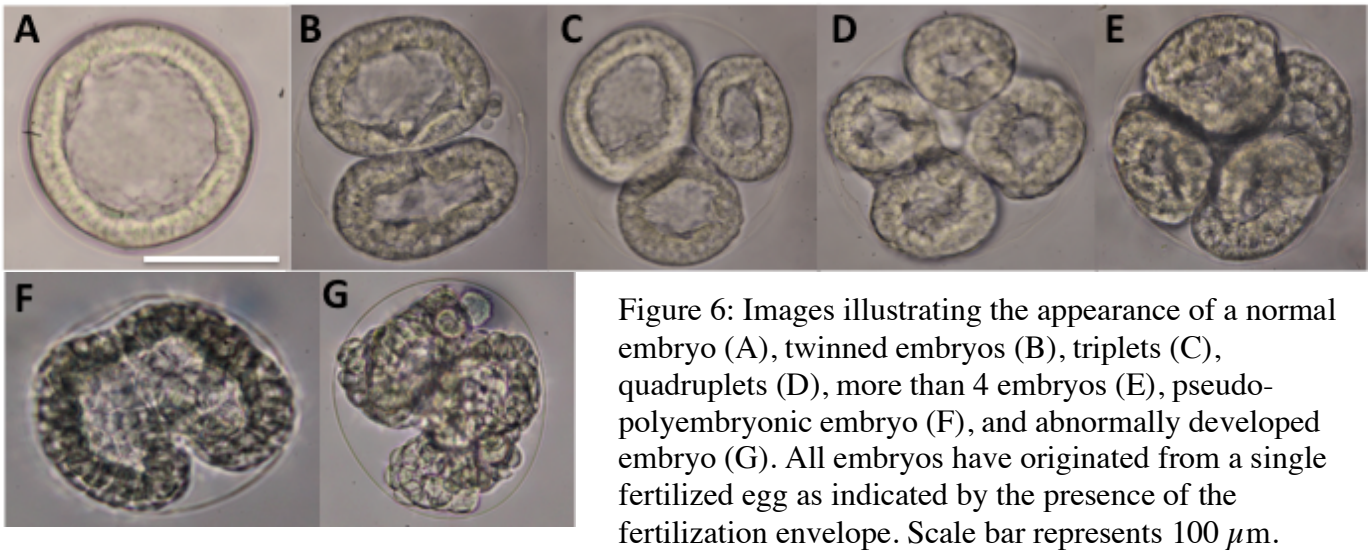


Figure 6: Images illustrating the appearance of a normal embryo (A), twinned embryos (B), triplets (C), quadruplets (D), more than 4 embryos (E), pseudo-polyembryonic embryo (F), and abnormally developed embryo (G). All embryos have originated from a single fertilized egg as indicated by the presence of the fertilization envelope. Scale bar represents 100 μm.

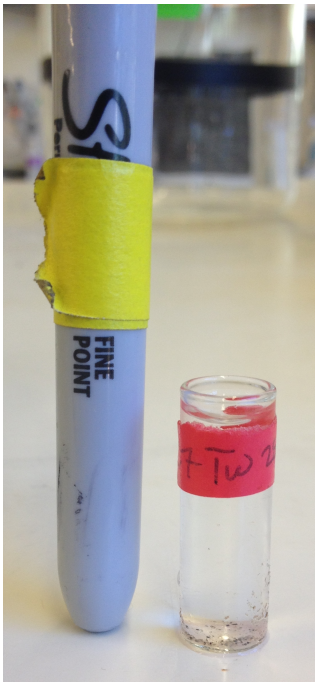


Figure 7: Image of a cuvette containing a unique set of twins, with a sharpie marker for scale.

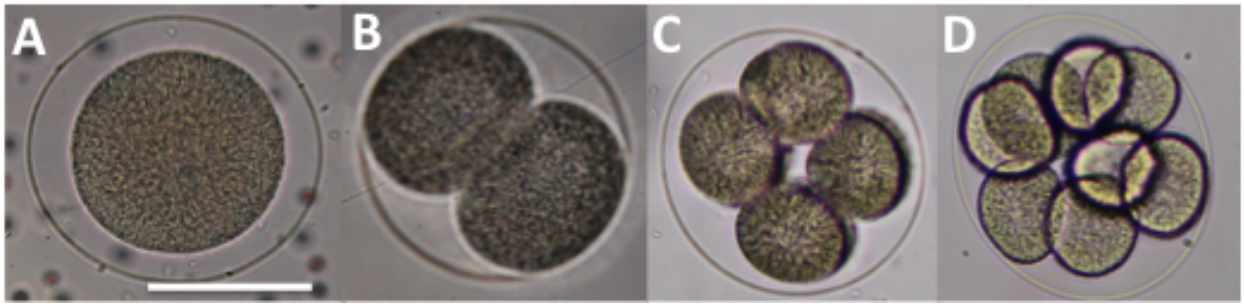


Figure 8: Images of the cleavage stages, one-cell (A), two-cell (B), four-cell (C), and 8+ cell (D), scored for in DOH experiments conducted in embryos of *D. excentricus*. Scale bar represents 100 μm.

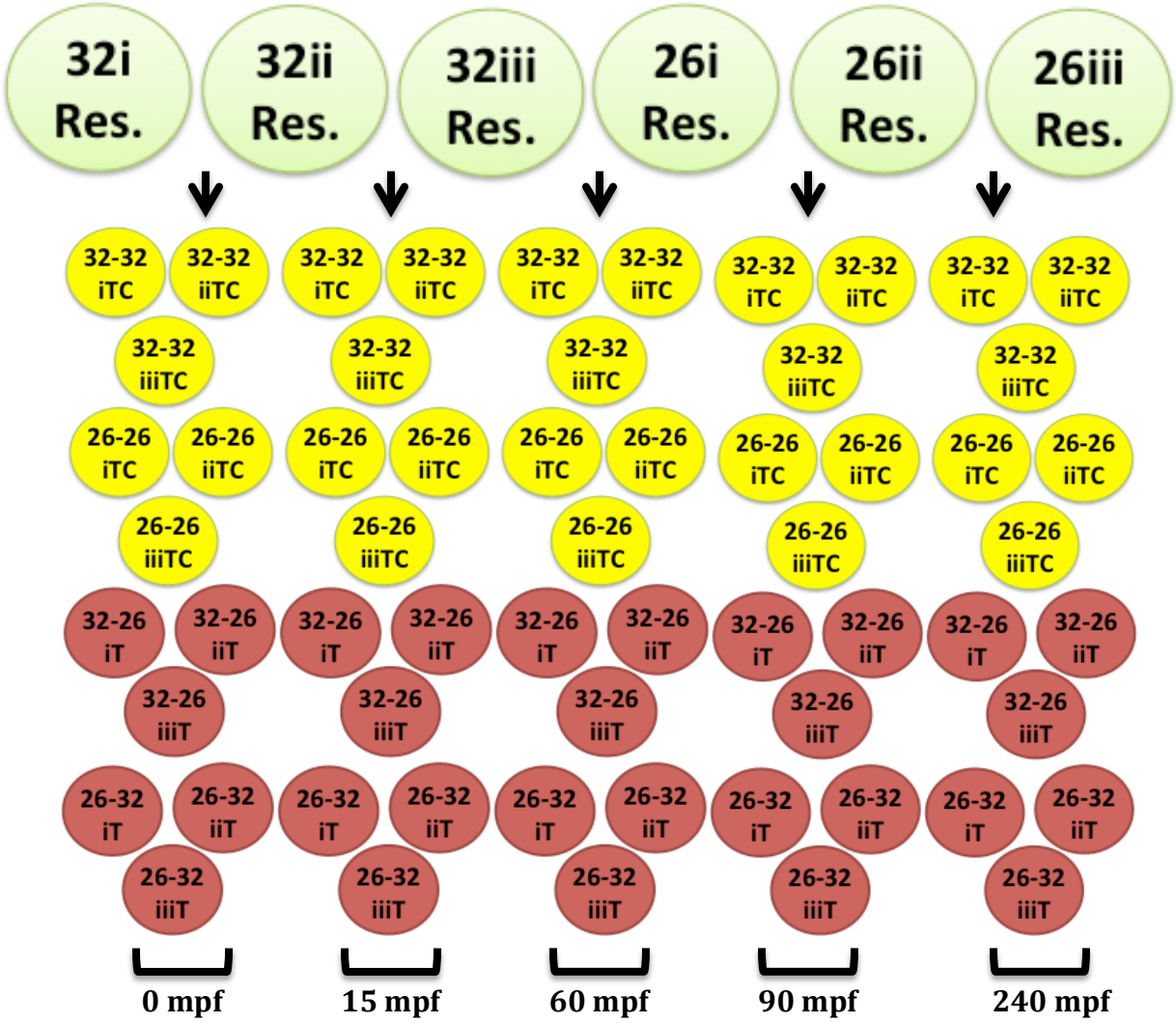


Figure 9: Schematic of the setup of the transfer experiments conducted on embryos of *D. excentricus*. Only 32 and 26 ppt salinity treatments were utilized for these experiments. Green circles indicate reservoir (Res.) bowls from which embryos were mouth pipetted out at the indicated time period (0, 15, 60, 90, and 240 minutes post-fertilization) and into the corresponding transfer (T) or transfer control (TC) bowls. Yellow circles indicate TC bowls and red circles indicate T bowls.

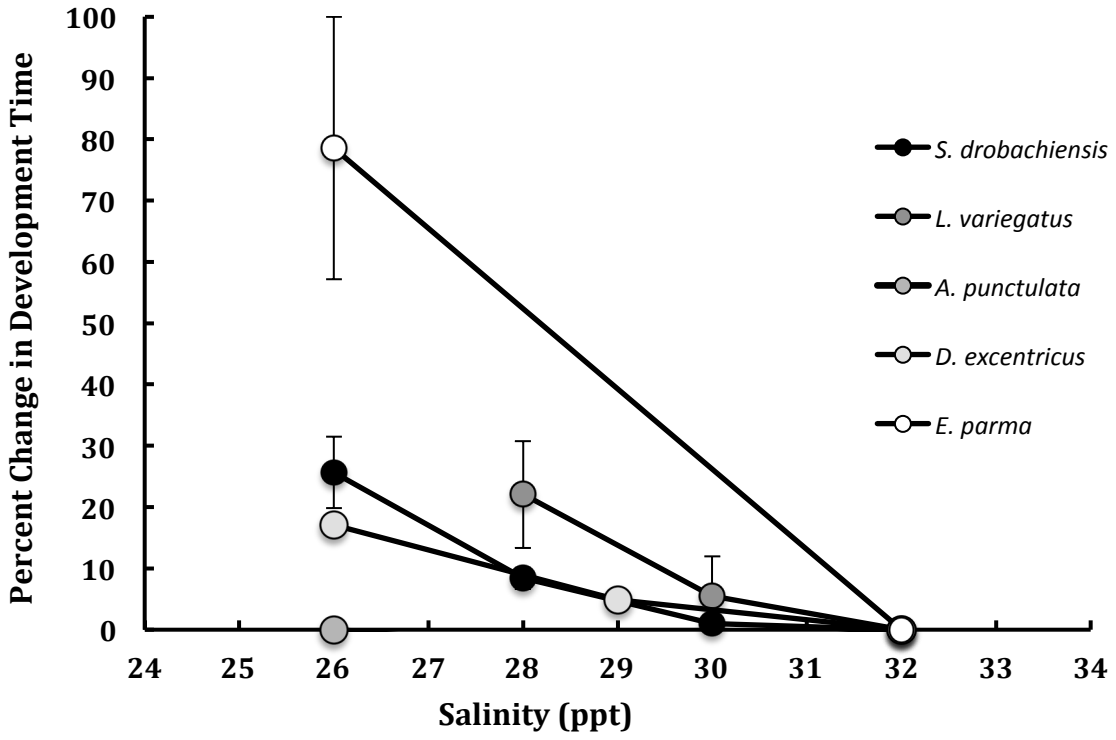


Figure 10: Cross-species comparison of time to hatching across salinities. Points indicate means ± standard error for three replicate parental pairs (n=3) for all species except for *E. parma* (n=2). Hatching data from *E. parma* was provided from experiments conducted by Armstrong *et al*. (2013). Both species identity and salinity treatment significantly affected the change in time to hatching (2-way ANOVA; $F_{4,14} = 35.133$; $p < 0.001$ and $F_{3,14} = 3.719$; $p = 0.037$ respectively) but there was no significant interaction between the two (2-way ANOVA, $F_{3,14} = 1.551$; $p = 0.242$). Bonferroni-corrected post-hoc comparisons revealed that the increased time to hatching for *E. parma* was significantly greater than for all other species ($p < 0.001$) and that values for *A. punctulata* were significantly lower than for all other species ($p < 0.05$).

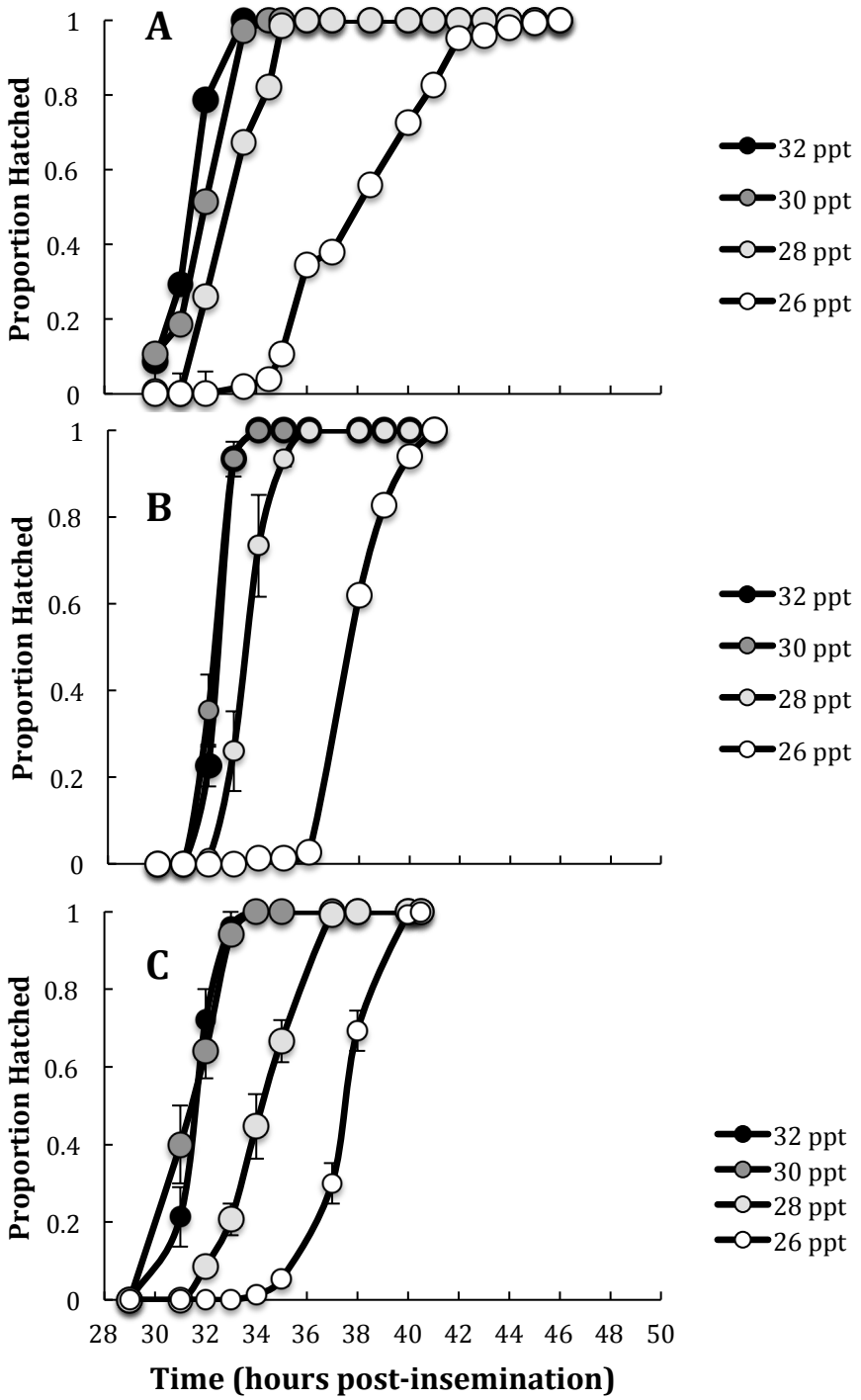


Figure 11: Hatching responses across salinities in embryos of *S. droebachiensis*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, dark grey indicates exposure to 30 ppt seawater, light grey circles indicate 28 ppt, and white circles correspond to embryos maintained in 26 ppt seawater. Salinity was found to have a significant ($p < 0.001$) effect on hatching in all three trials.

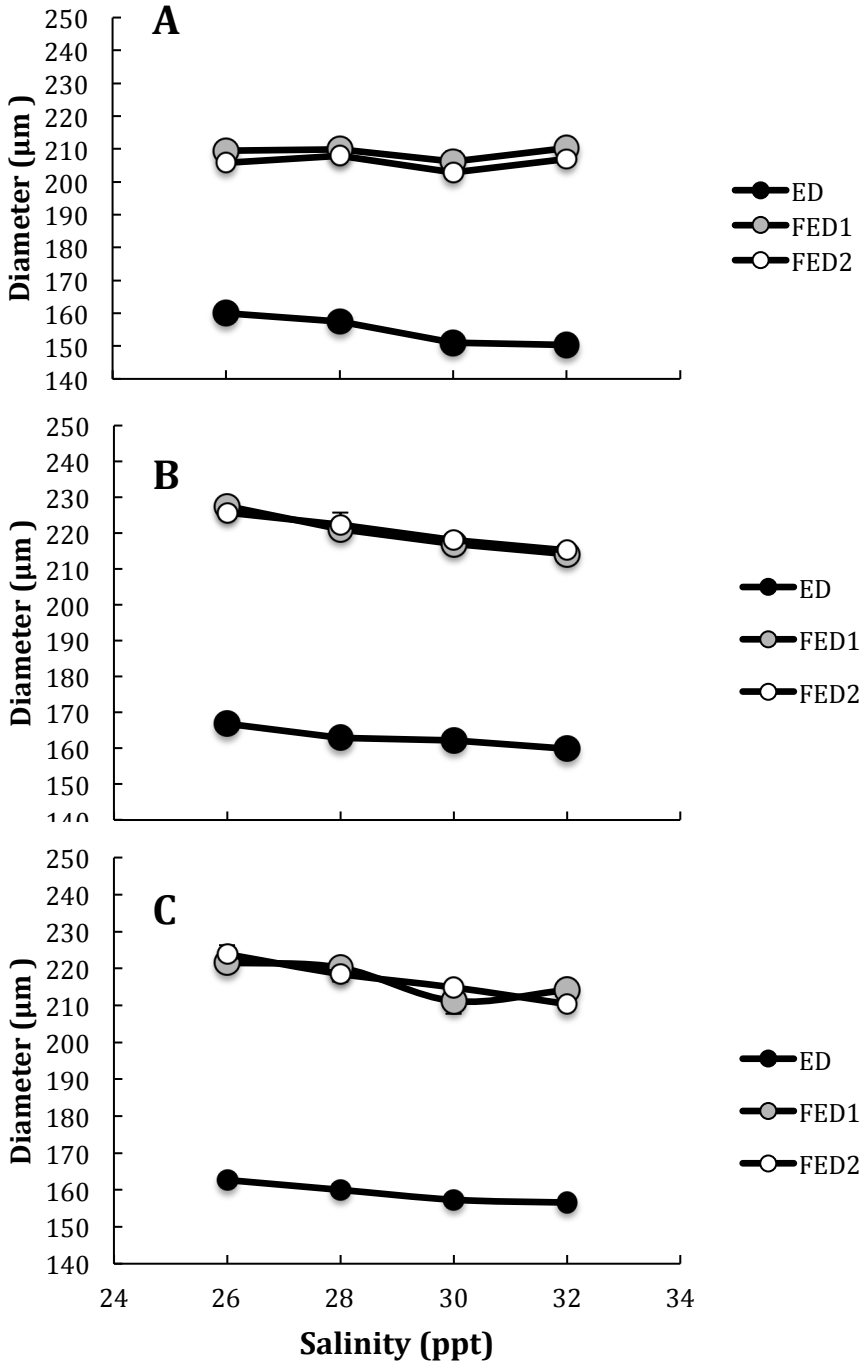


Figure 12: Egg diameter (ED), initial fertilization envelope diameter (FED1), and pre-hatching fertilization envelope diameter (FED2) measurements across salinities in embryos of *S. droebachiensis*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles represent FED1 measurements, and white circles correspond to FED2 measurements. Salinity was found to have a significant ($p < 0.001$) effect on ED and FED, however, time (between FED1 and FED2 measurements) and its interaction with salinity was not found to have a significant effect on FED ($p > 0.050$).

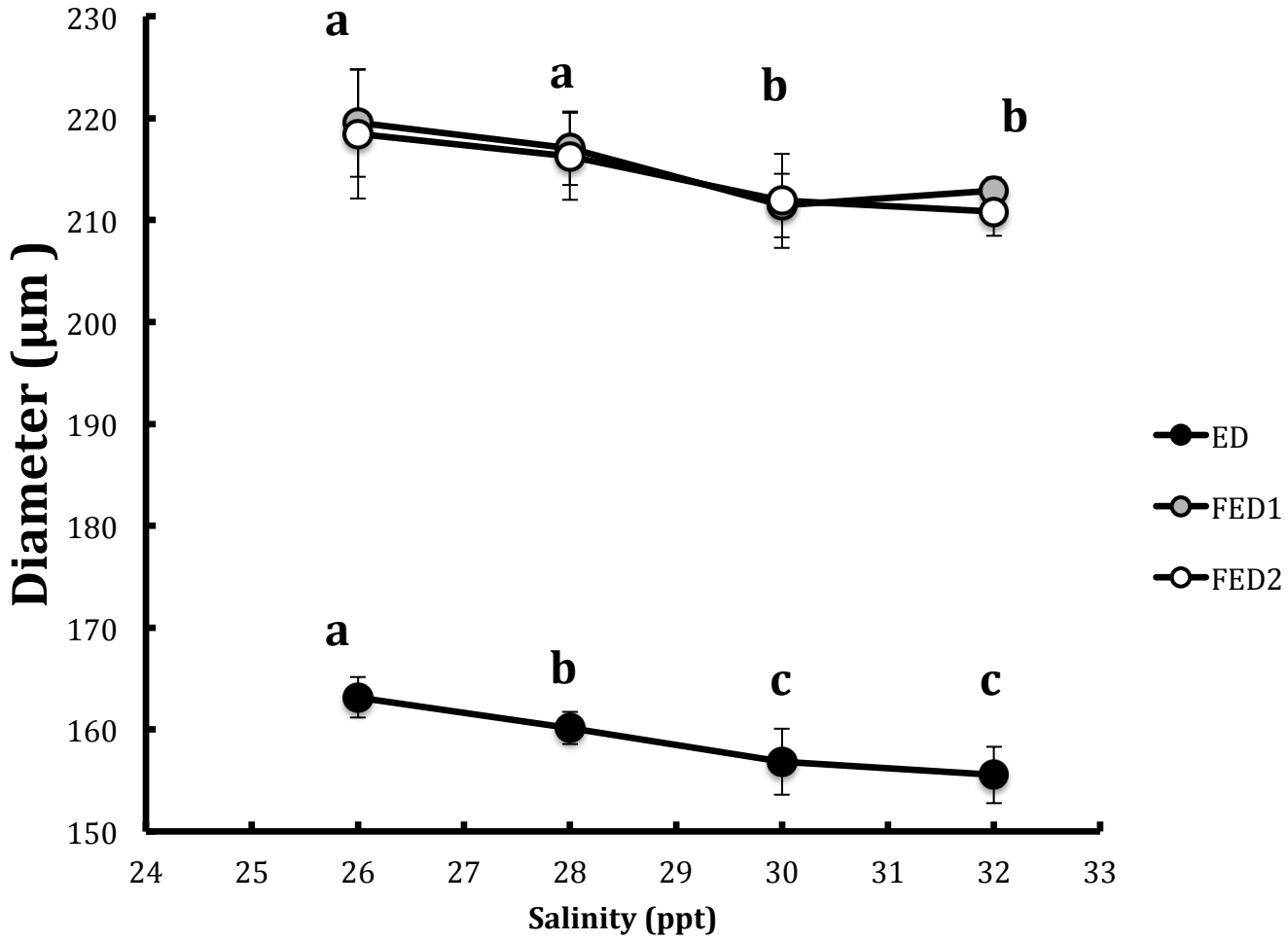


Figure 13: ED, FED1, and FED2 measurements of *S. droebachiensis* embryos from the three aforementioned experimental trials. Each point is the mean ± standard error from three replicate experiments. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between different salinity treatments, whereas similar letters indicate non-significant differences.

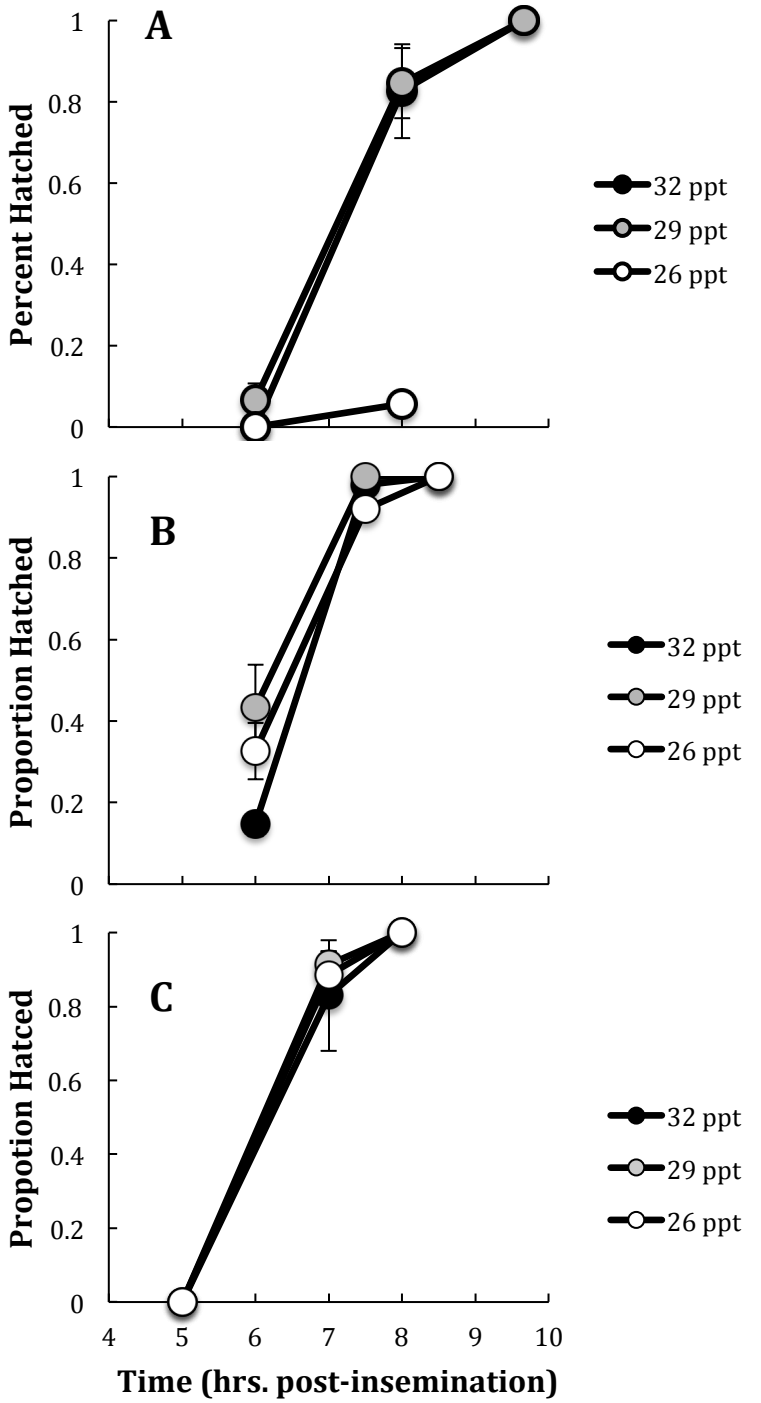


Figure 14: Hatching responses across salinities in embryos of *A. punctulata*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Salinity was found to have a significant ($p < 0.001$) effect on hatching in trials 1 and 2, but not trial 3 ($p > 0.900$). This discrepancy is due to the lack of hatching variation in the initial time point, which interfered with the capabilities of the analysis to adequately interpret the data.

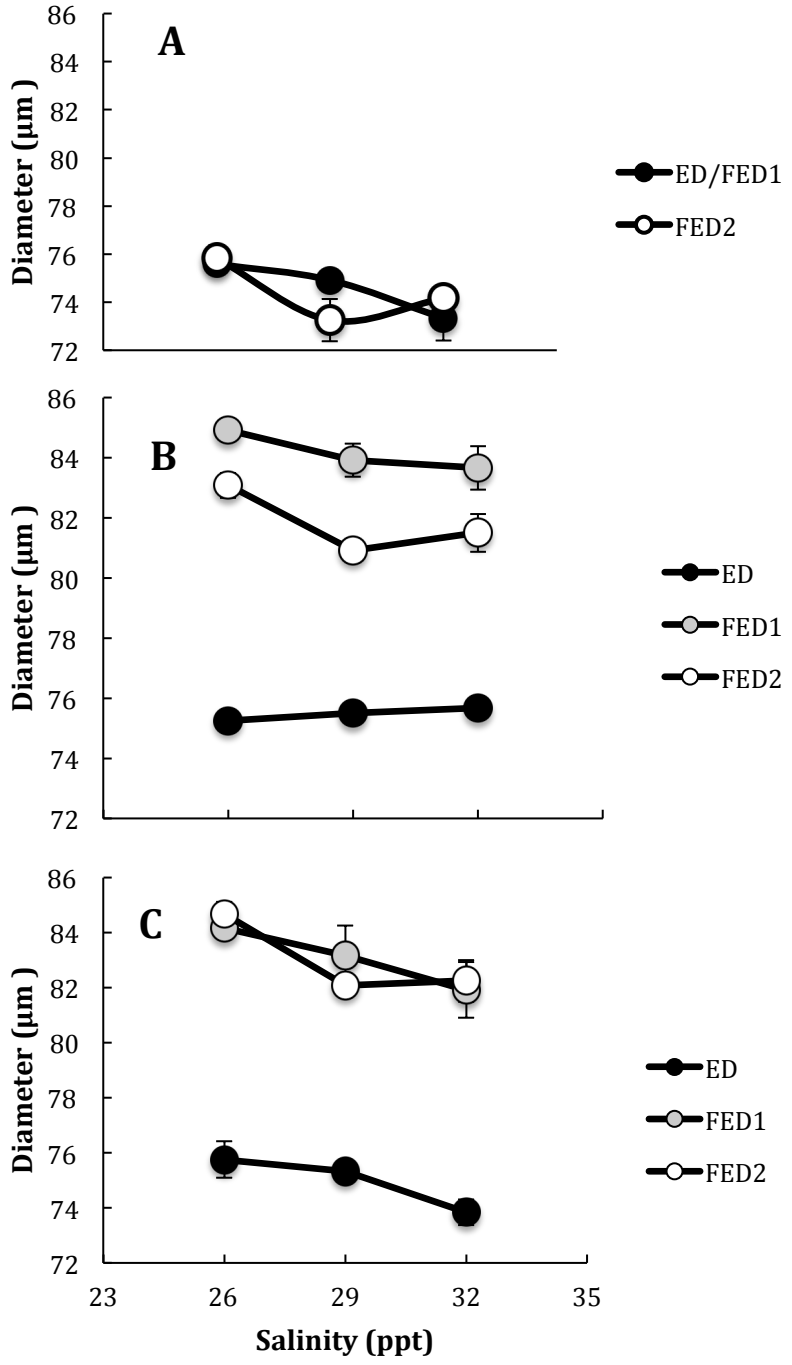


Figure 15: ED, FED1, and FED2 measurements across salinities in embryos of *A. punctulata*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles represent FED1 measurements, and white circles correspond to FED2 measurements. ED and FED1 are plotted on the same line in the first experimental trial due the unappreciable rise of the FE around the egg, making it difficult to discern between the egg and FE. Salinity was found to have a significant ($p < 0.001$) effect on ED. Additionally, salinity and time were found to have a significant effect on FED ($p < 0.050$), but their interaction was not significant ($p > 0.050$).

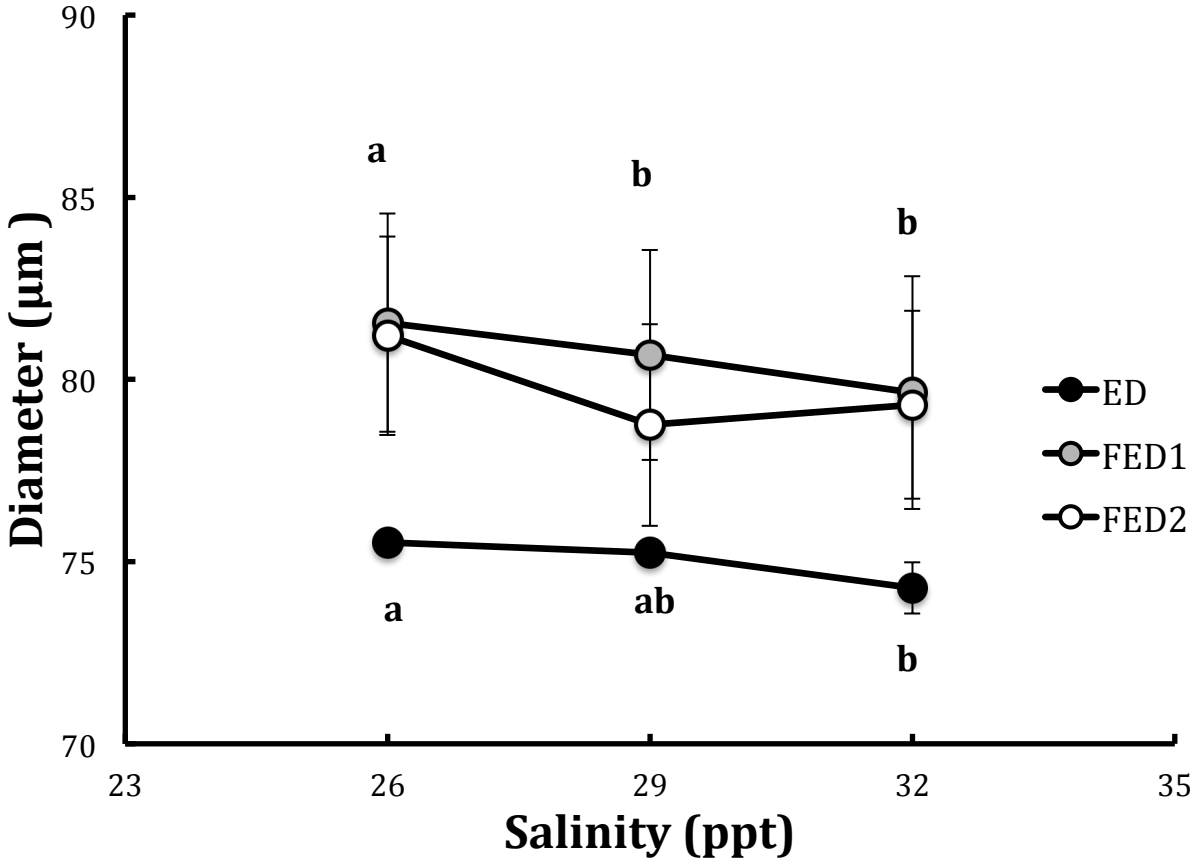


Figure 16: ED, FED1, and FED2 measurements of *A. punctulata* embryos from the three aforementioned experimental trials. Each point is the mean ± standard error for three replicate experiments. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between salinity treatments, whereas similar letters indicate non-significant measurements. The "ab" designation under the 29 ppt mean ED point is due to non-significant differences between the 29 ppt and the 26 and 32 ppt treatments ($p > 0.050$), however, 26 and 32 ppt treatments were both found to be significantly different from one another ($p < 0.050$).

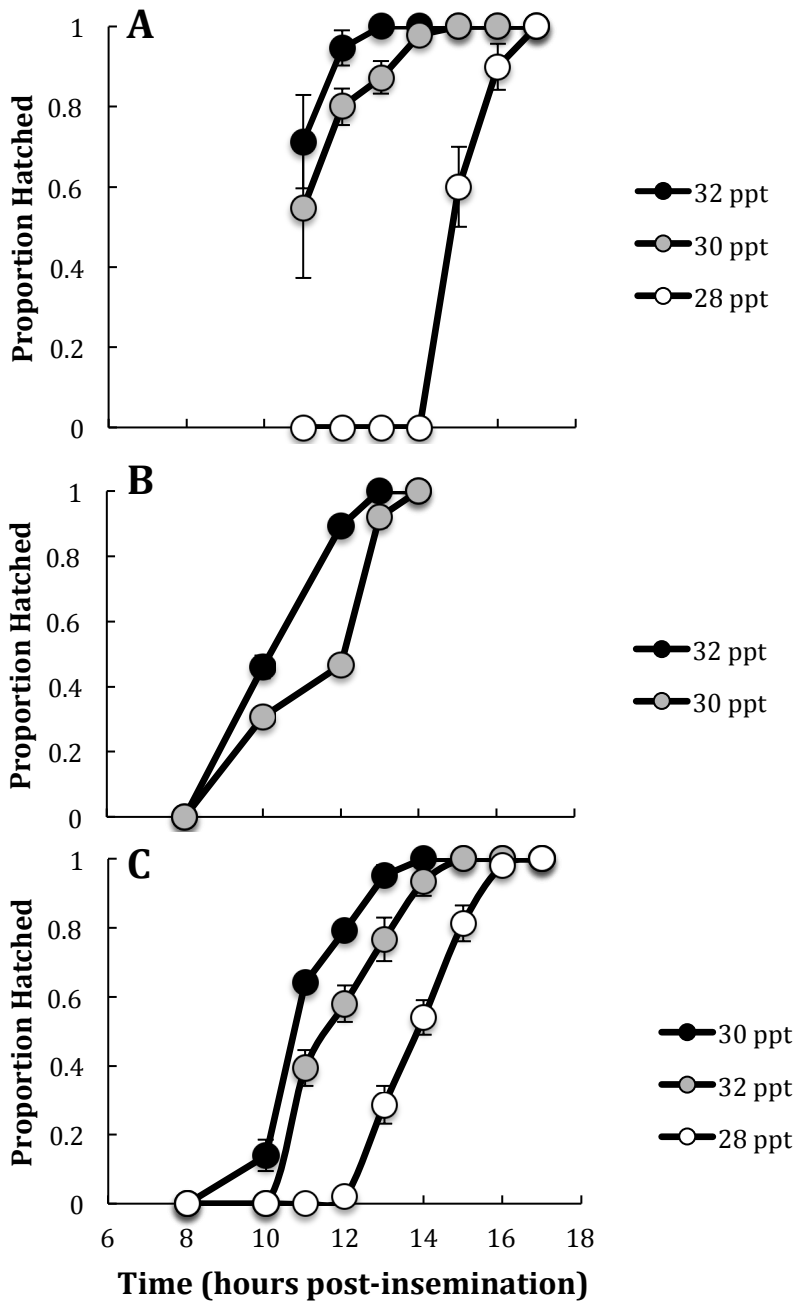


Figure 17: Hatching responses across salinities in embryos of *L. variegatus*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 30 ppt seawater, and white circles correspond to embryos maintained in 28 ppt seawater. Embryos exposed to 26 ppt seawater were consistently observed to fail to develop properly, and so those data are omitted. Additionally, embryos exposed to 28 ppt seawater in trial 2 did not develop normally in high proportions and data for that salinity treatment in that trial are also omitted. Salinity was found to have a significant ($p < 0.001$) effect on hatching in all trialsp < 0.001).

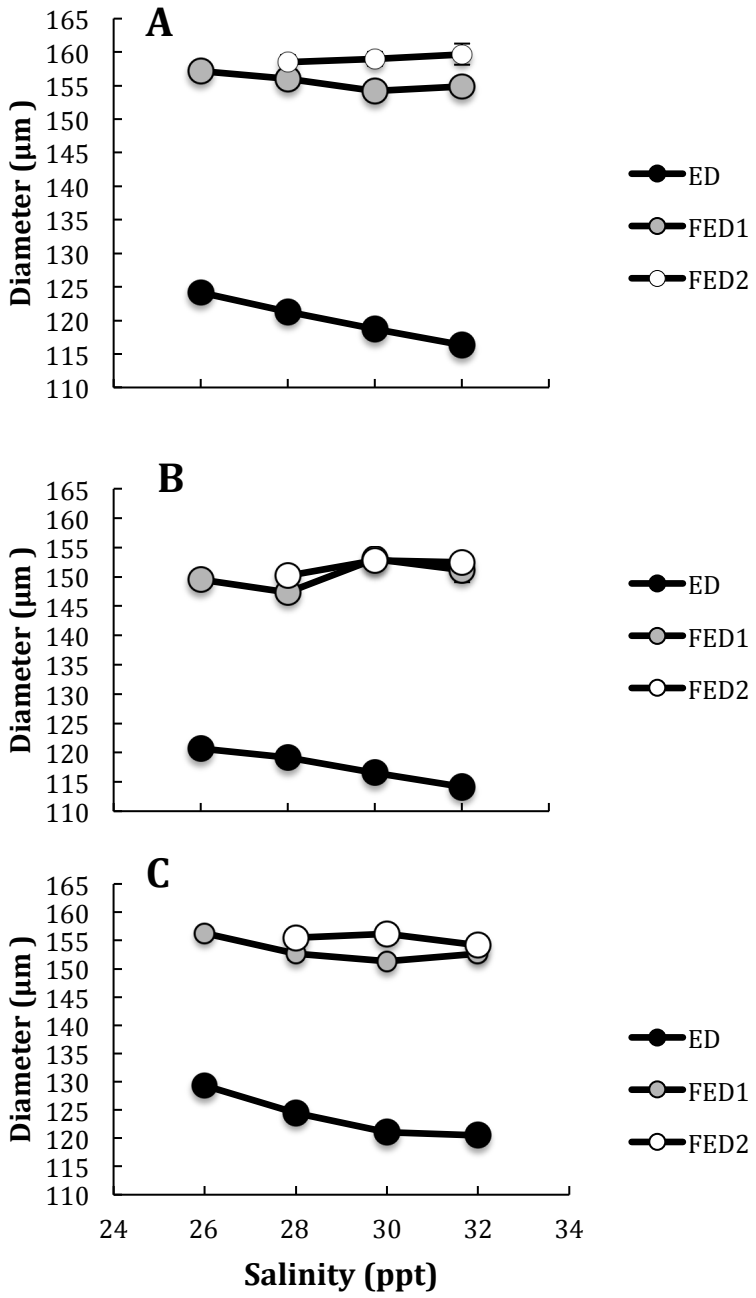


Figure 18: ED, FED1, and FED2 measurements across salinities in embryos of *L. variegatus*. Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles represent FED1 measurements, and white circles correspond to FED2 measurements. FED2 data for embryos exposed to 26 ppt seawater are omitted due to the embryos' inability to undergo normal development under that salinity treatment. Salinity was found to have a significant ($p < 0.001$) effect on ED, but it did not have a significant effect on FED1 ($p > 0.050$). Time was found to have a significant effect on FED ($p < 0.050$), but its interaction with salinity was not significant ($p > 0.050$).

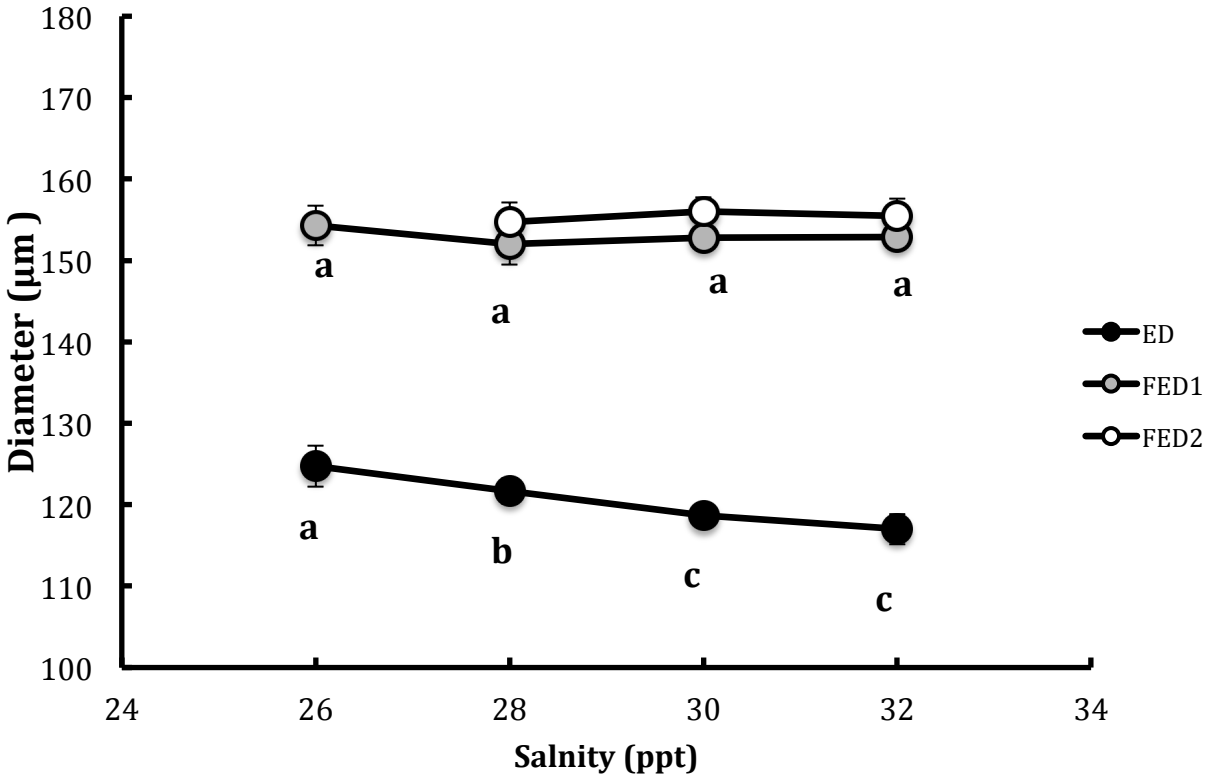


Figure 19: ED, FED1, and FED2 measurements of *L. variegatus* embryos from the three aforementioned experimental trials. Each point is the mean ± standard error for three replicate experiments. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between salinity treatments, whereas similar letters indicate non-significant measurements. All salinity treatments were not found to be significant from one another with regards to FED($p > 0.050$), however, 26 and 28 ppt treatments were both found to be significantly different from one another, 30, and 32 ppt treatments ($p < 0.050$), whereas the latter two treatments were not significantly different from one another ($p > 0.050$).

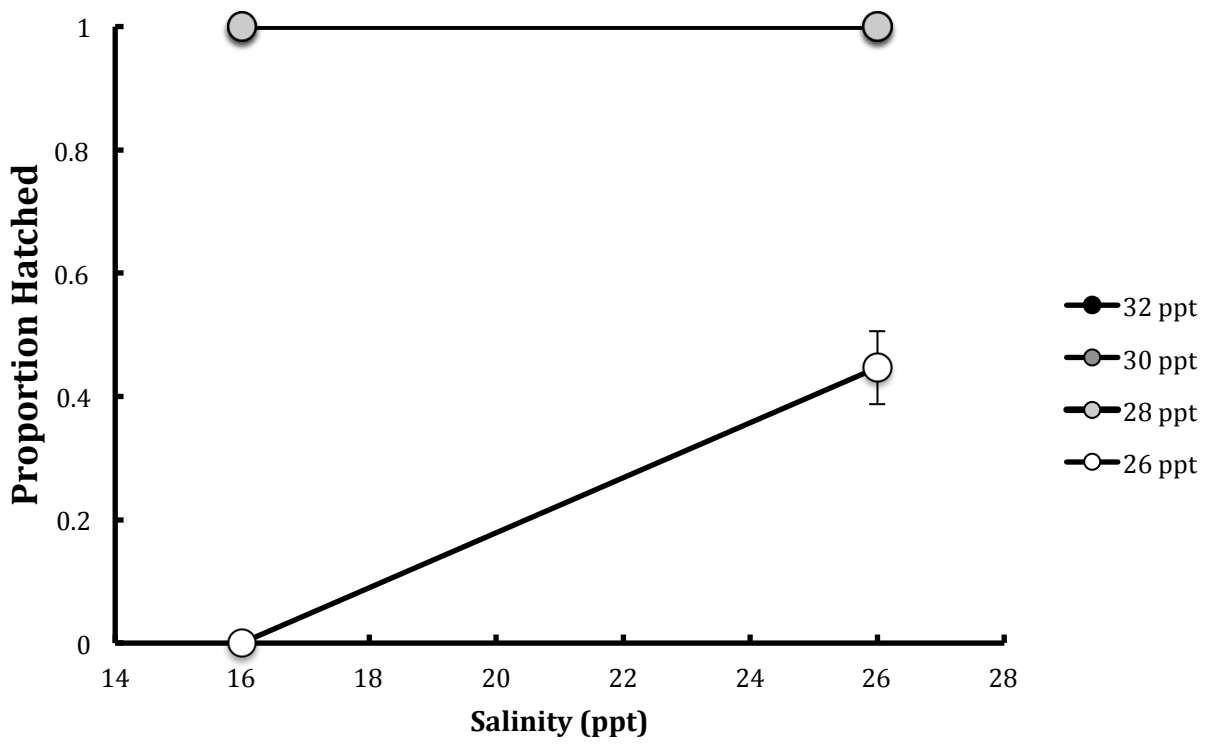


Figure 20: Hatching responses across salinities in embryos of *E. tribuloides*. Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, dark grey indicates exposure to 30 ppt seawater, light grey circles represent embryos exposed to 28 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Embryos were scored for hatching at 16 hpf and at 26 hpf. Embryos in 32, 30, and 28 ppt treatments were observed to have reached 100 % hatching at 16 hours, whereas embryos in the 26 ppt treatment had not yet begun to hatch. At approximately 26 hpf, embryos in 26 ppt seawater were observed to have reached nearly 50 % hatching. These data are from a single experiment with only one parental pair used.

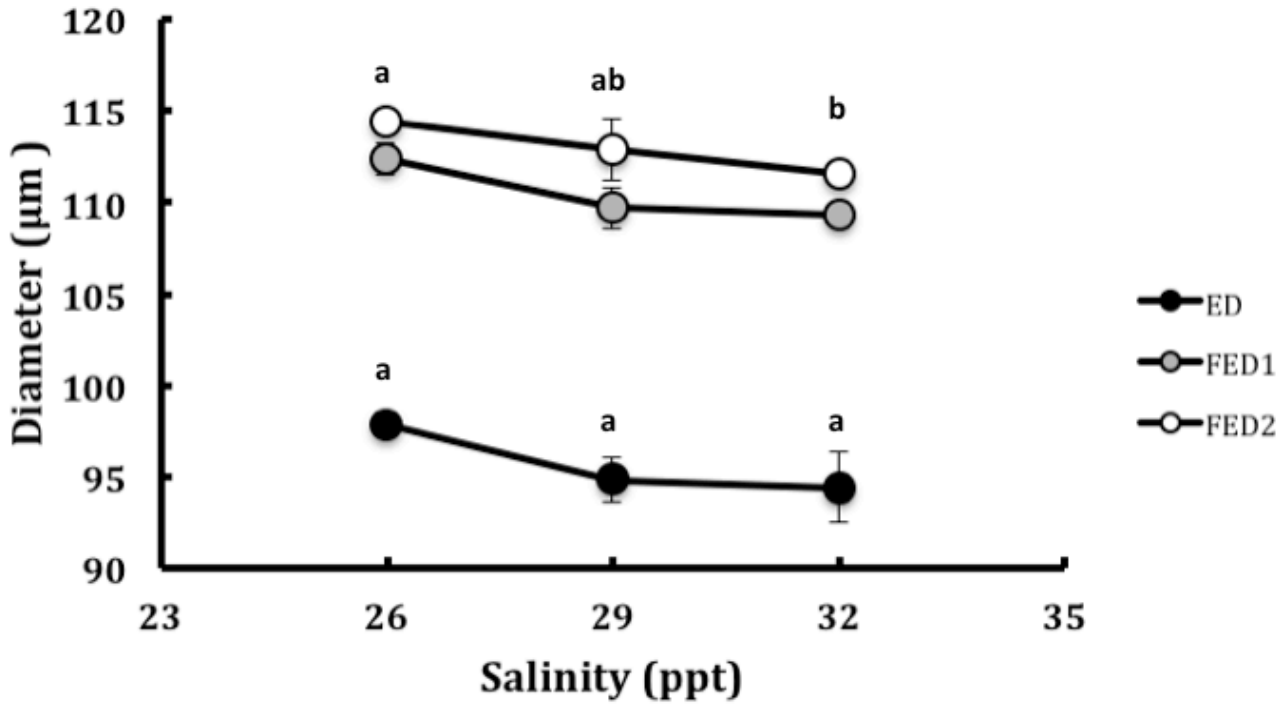


Figure 21: ED, FED1, and FED2 measurements of *E. tribuloides* embryos from a single experimental trial. Each point is the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between salinity treatments, whereas similar letters indicate non-significant measurements. All salinity treatments were not found to be significant from one another with regards to ED($p > 0.050$). The "ab" designation above the 29 ppt mean FED points is due to non-significant differences between the 29 ppt and the 26 and 32 ppt treatments ($p > 0.050$), however, 26 and 32 ppt treatments were both found to be significantly different from one another ($p < 0.050$).

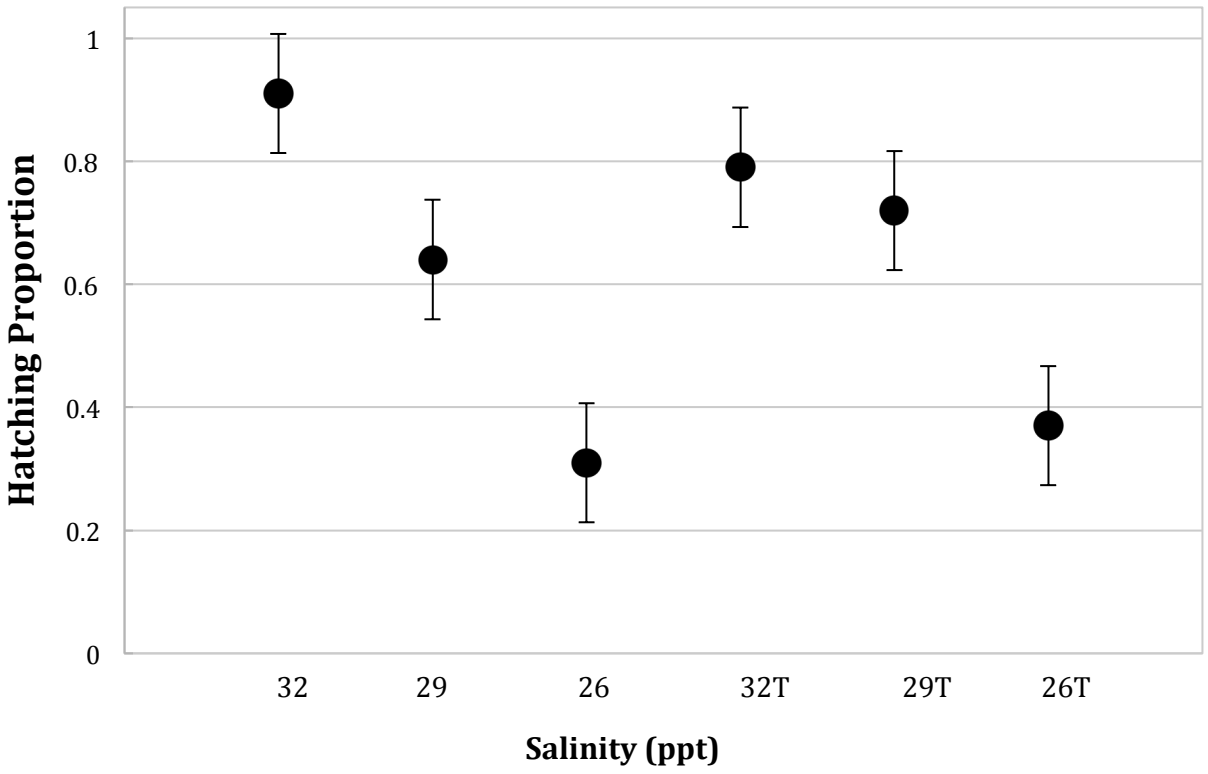


Figure 22: Hatching responses across salinities in embryos of *E. parma*. Each point represents the mean ± standard error for three replicate bowls. Transfer treatments, 32T, 29T, and 26T, represent embryos that were transferred from an initial salinity treatment of 32, 29, or 26 ppt and into full strength seawater (32 ppt) at approximately 7 hpf. Embryos initially fertilized in either 26 or 29 ppt seawater were observed to have a 0.08 increase in hatching proportion in comparison to non-transfer treatments. Conversely, embryos that were control transferred from 32 to 32 ppt seawater exhibited a 0.12 decrease in hatching proportion.

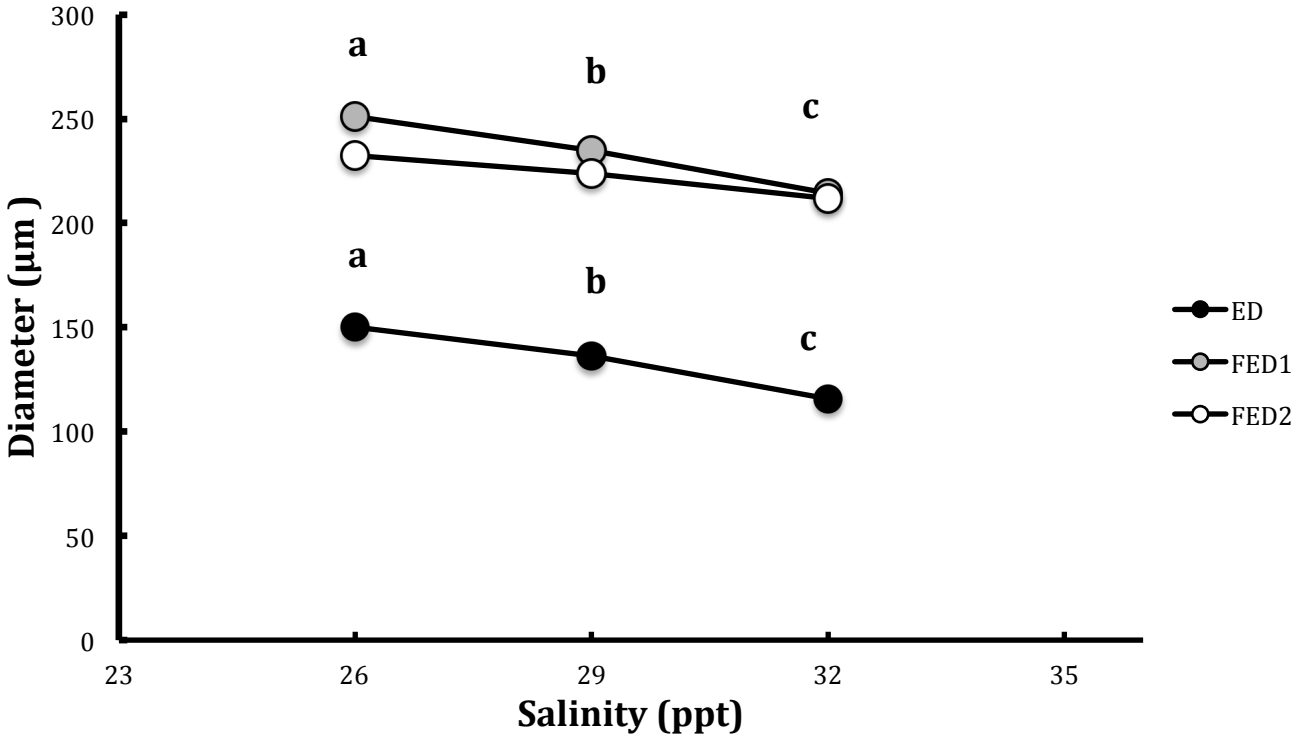


Figure 23: ED, FED1, and FED2 measurements of *E. parma* embryos from a single experimental trial. Each point is the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between salinity treatments, whereas similar letters indicate non-significant measurements. All salinity treatments were not found to be significant from one another ($p < 0.050$).

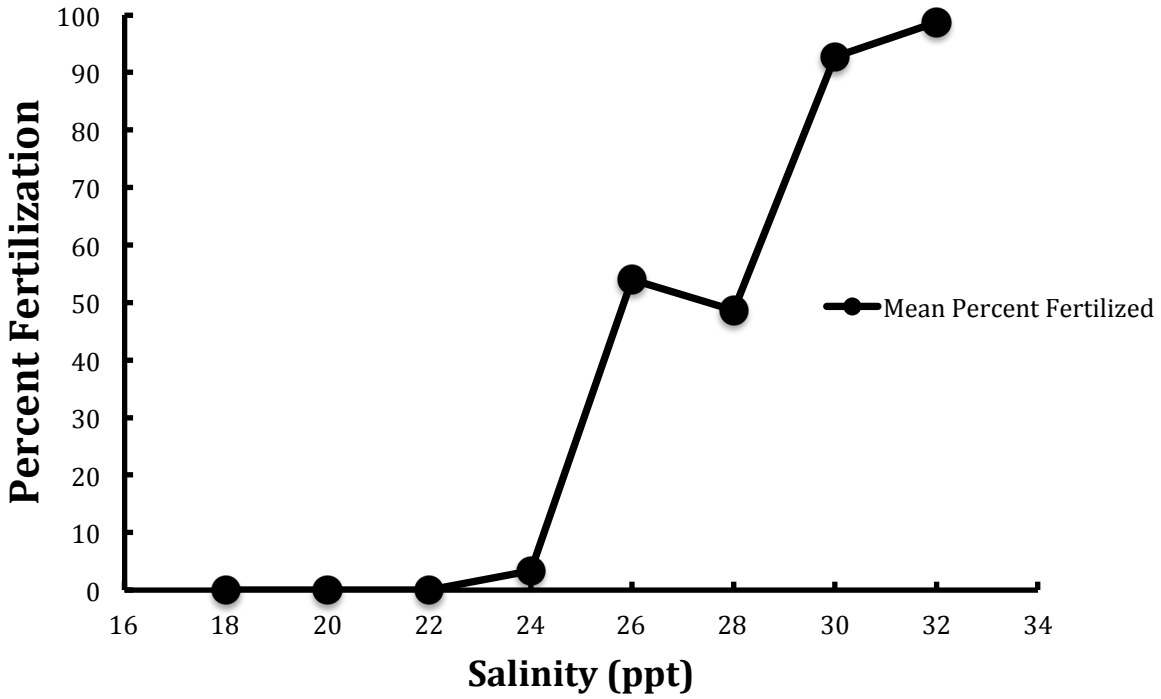


Figure 24: Fertilization assay conducted on embryos of *D. excentricus* in which salinity treatments ranged from 32 to 18ppt. Embryos were observed to fail to fertilize in proportions equal or greater than 0.500 at salinities below 26 ppt, and so that was the salinity used for further experimentation.

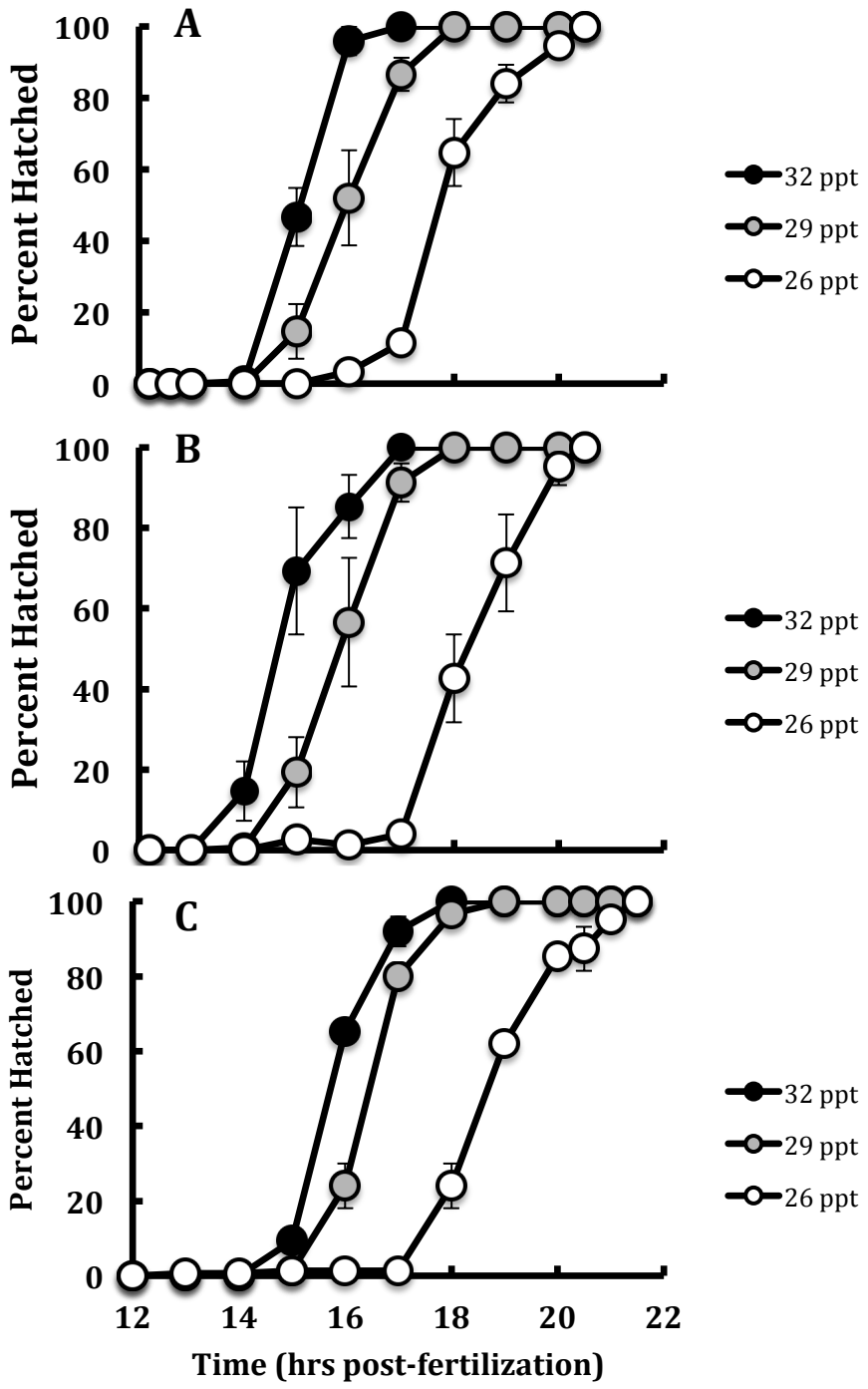


Figure 25: Hatching responses across salinities in embryos of *D. excentricus* exposed to ambient water temperatures (12-15°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Salinity was found to have a significant ($p < 0.001$) effect on hatching in all trials ($p < 0.001$).

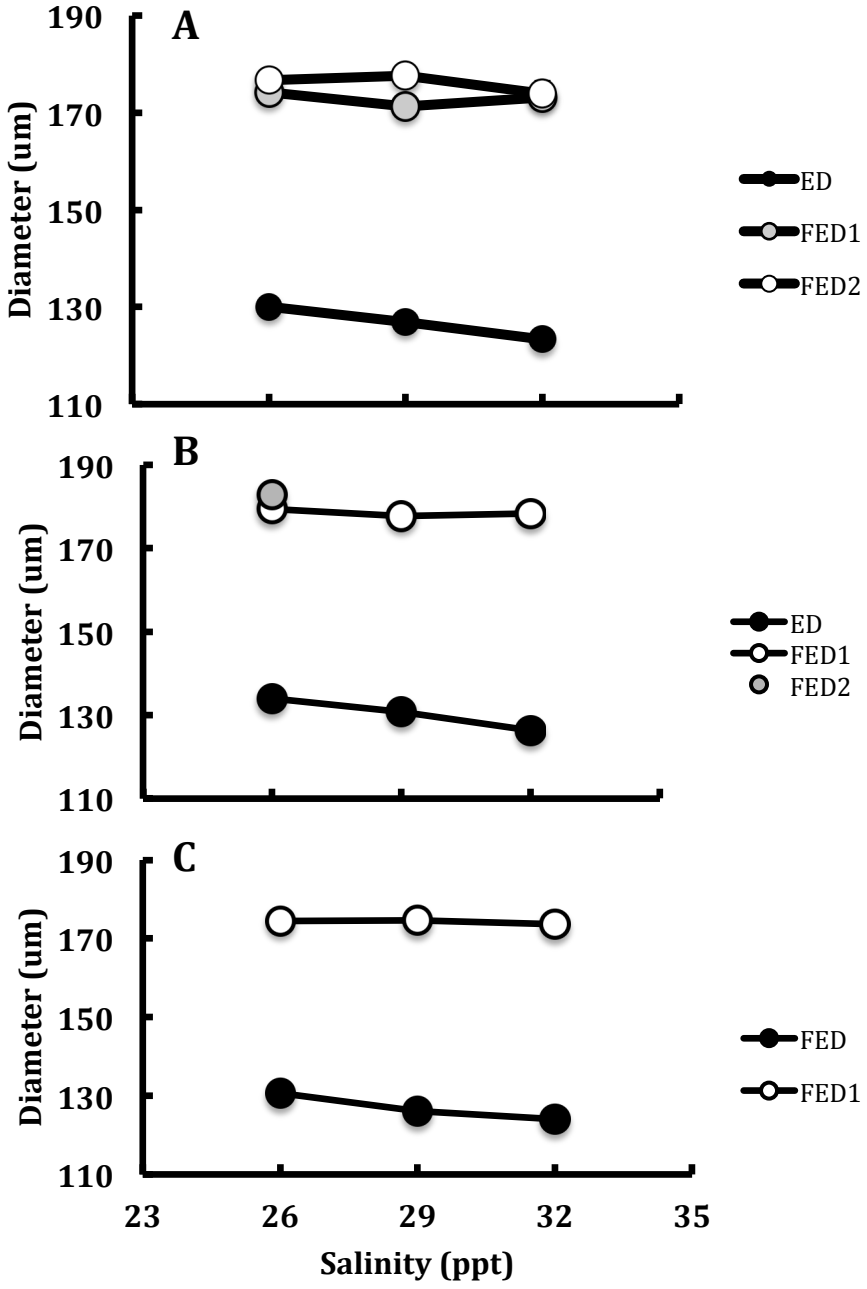


Figure 26: ED, FED1, and FED2 measurements across salinities in embryos of *D. excentricus* exposed to ambient water temperatures (12-15°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles represent FED1 measurements, and white circles correspond to FED2 measurements. FED2 data was only collected for embryos that produced multiples. Salinity was found to have a significant ($p < 0.001$) effect on ED, but it did not have a significant effect on FED ($p > 0.050$). Temperature was also found not to have a significant effect on FED ($p > 0.050$), however time was found to have a significant effect on FED ($p < 0.050$).

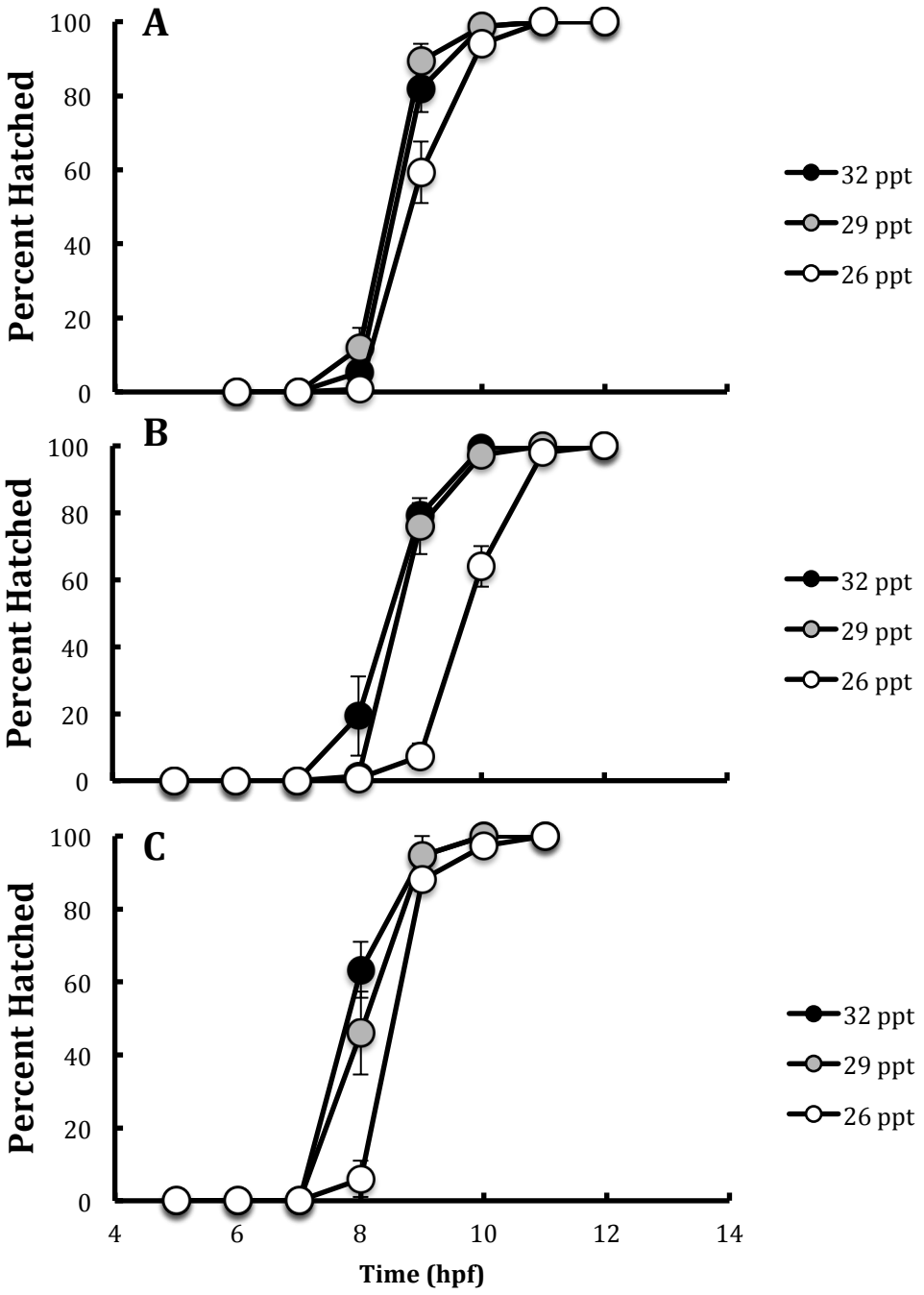


Figure 27: Hatching responses across salinities in embryos of *D. excentricus* exposed to heated water temperatures (19-23°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Salinity was found to have a significant ($p < 0.001$) effect on hatching in all trials ($p < 0.001$).

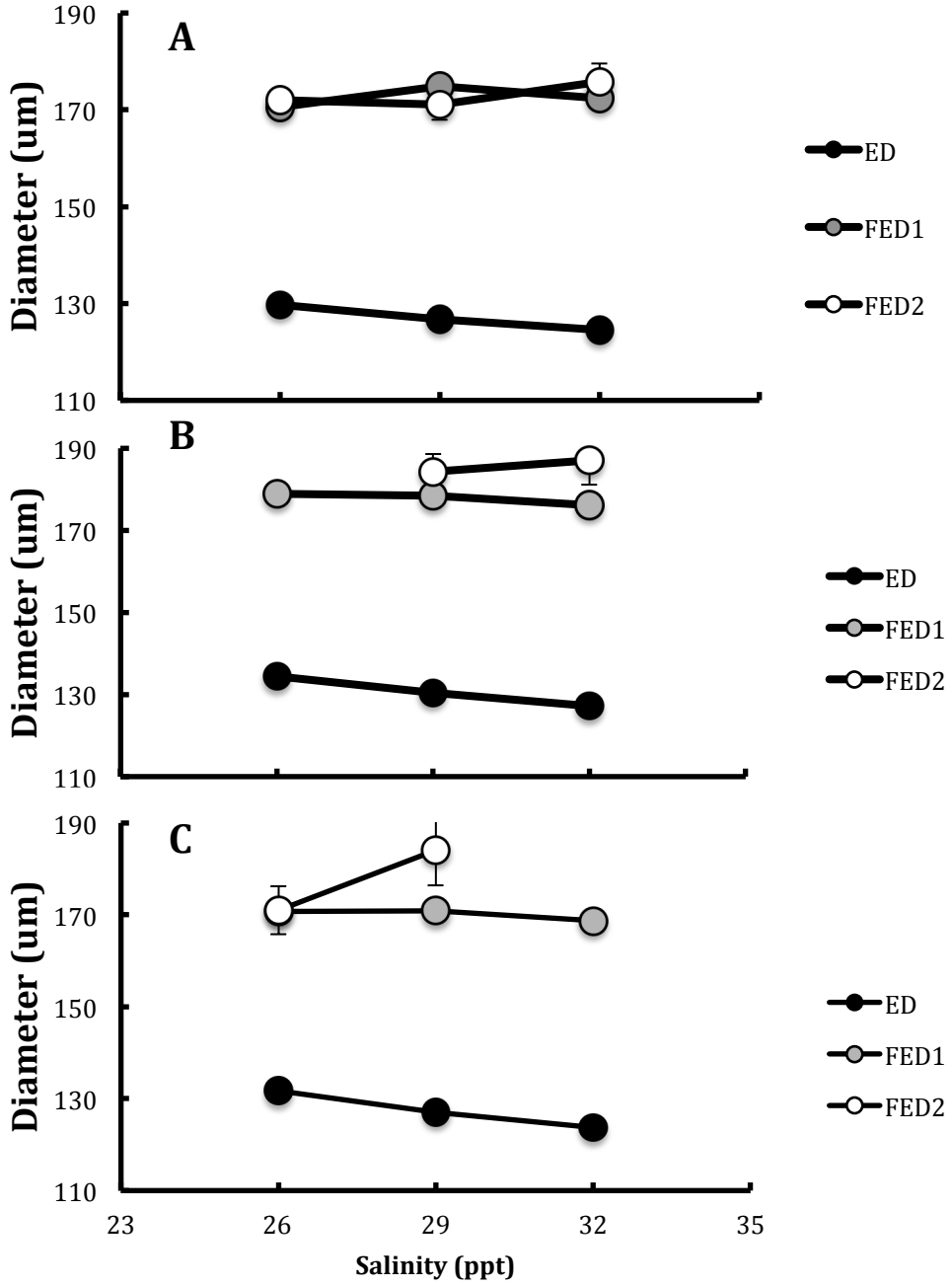


Figure 28: ED, FED1, and FED2 measurements across salinities in embryos of *D. excentricus* exposed to heated water temperatures (19-23°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate ED measurements, grey circles represent FED1 measurements, and white circles correspond to FED2 measurements. FED2 data was only collected for embryos that produced multiples. Salinity was found to have a significant ($p < 0.001$) effect on ED, but it did not have a significant effect on FED ($p > 0.050$). Temperature was also found not to have a significant effect on FED ($p > 0.050$), however time was found to have a significant effect on FED ($p < 0.050$).

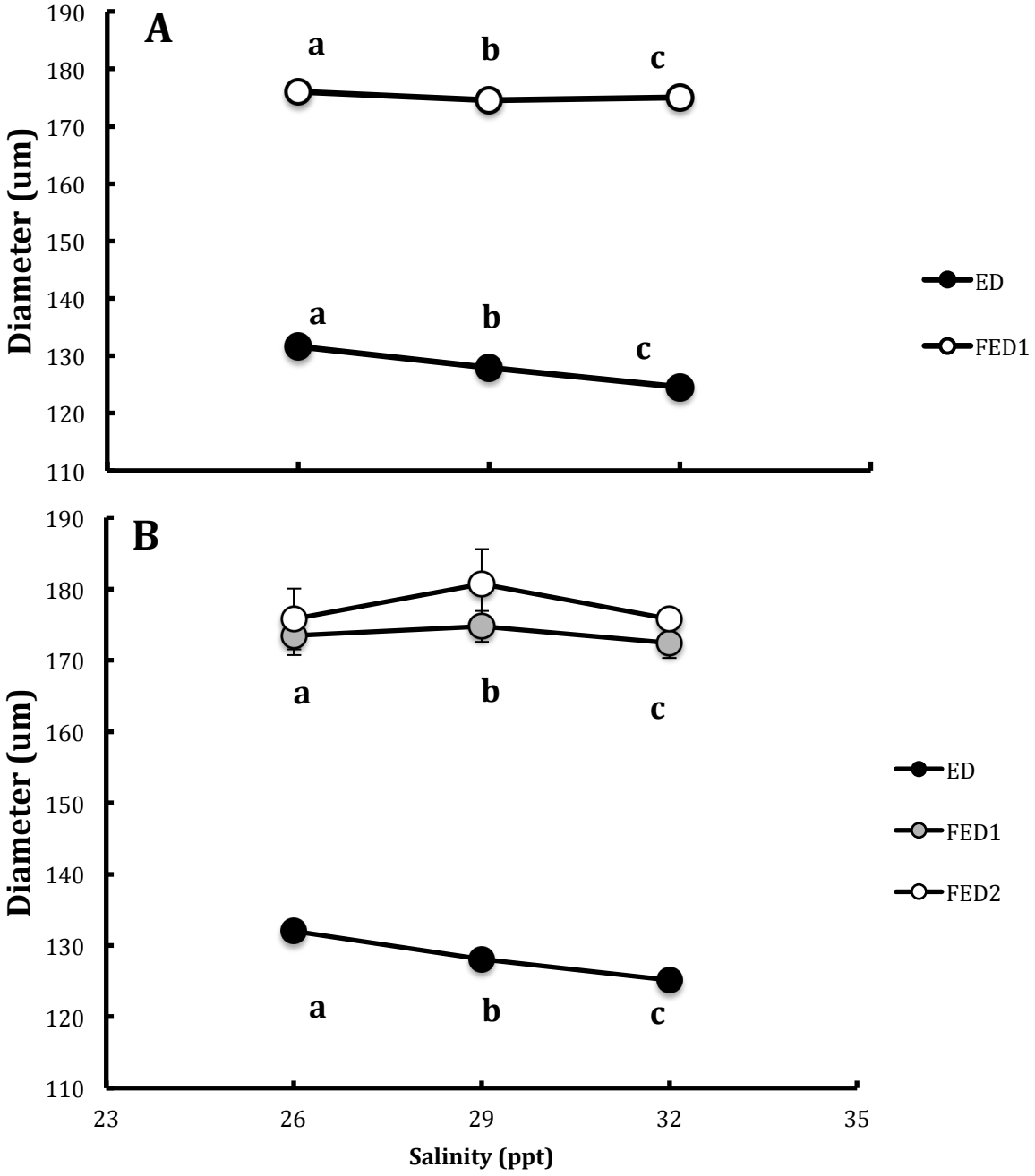


Figure 29: ED, FED1, and FED2 measurements in embryos of *D. excentricus* exposed to ambient water temperatures (A; 12-15°C), and heated water temperatures (B; 19-23°C) from the three aforementioned experimental trials. Each point is the mean ± standard error for three replicate experiments. Black circles indicate ED measurements, grey circles indicate FED1 measurements, and white circles correspond to FED2 measurements. Lowercase letters indicate results of post-hoc, Bonferroni corrected pairwise comparisons. Different letters indicate significant differences in diameter measurements between salinity treatments, whereas similar letters indicate non-significant measurements. All salinity treatments were found to be significant from one another with regards to both ED and FED($p < 0.050$).

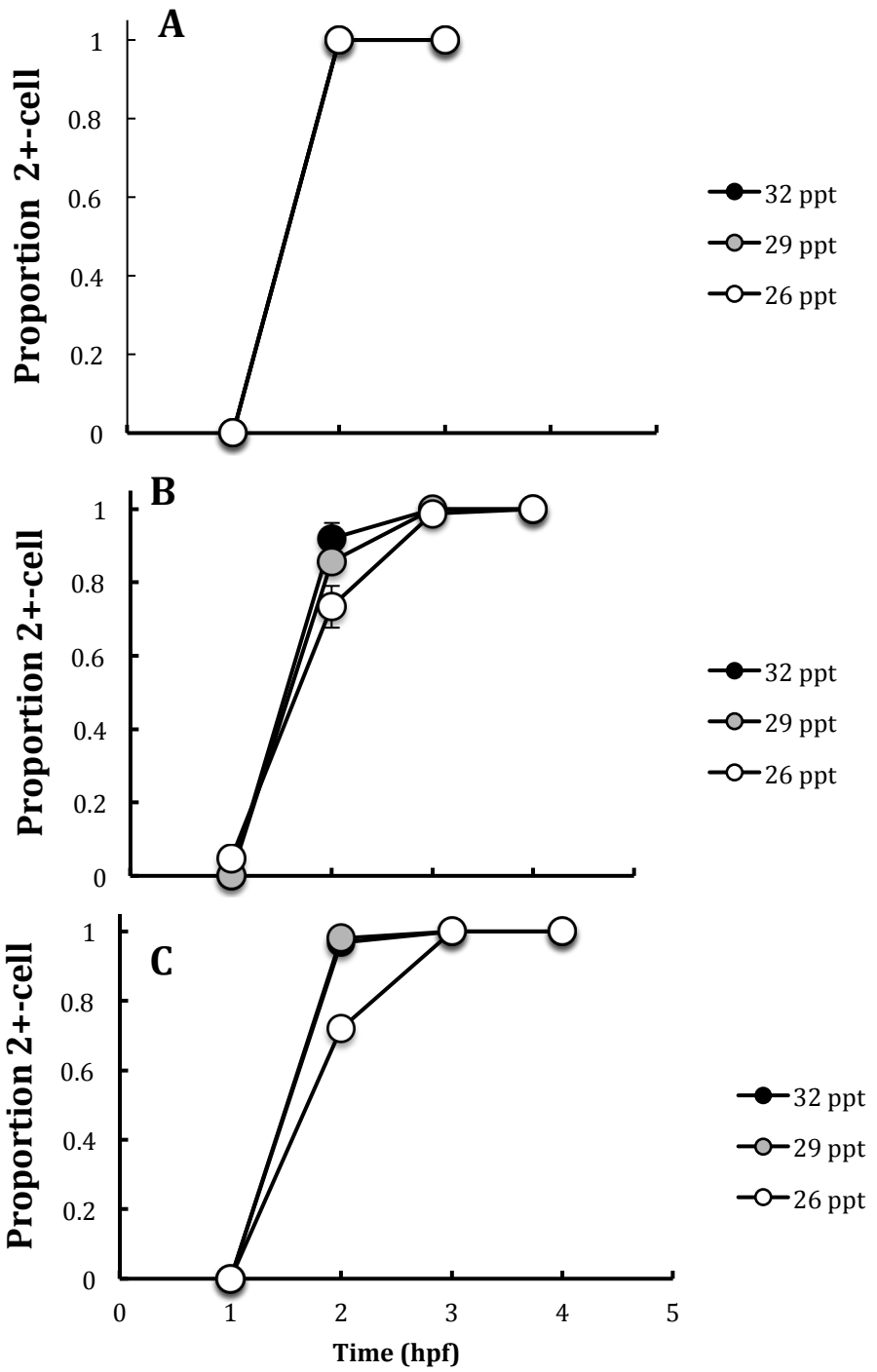


Figure 30: Cleavage to the 2+ cell stage in embryos of *D. excentricus* exposed to ambient water conditions (12-15°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$).

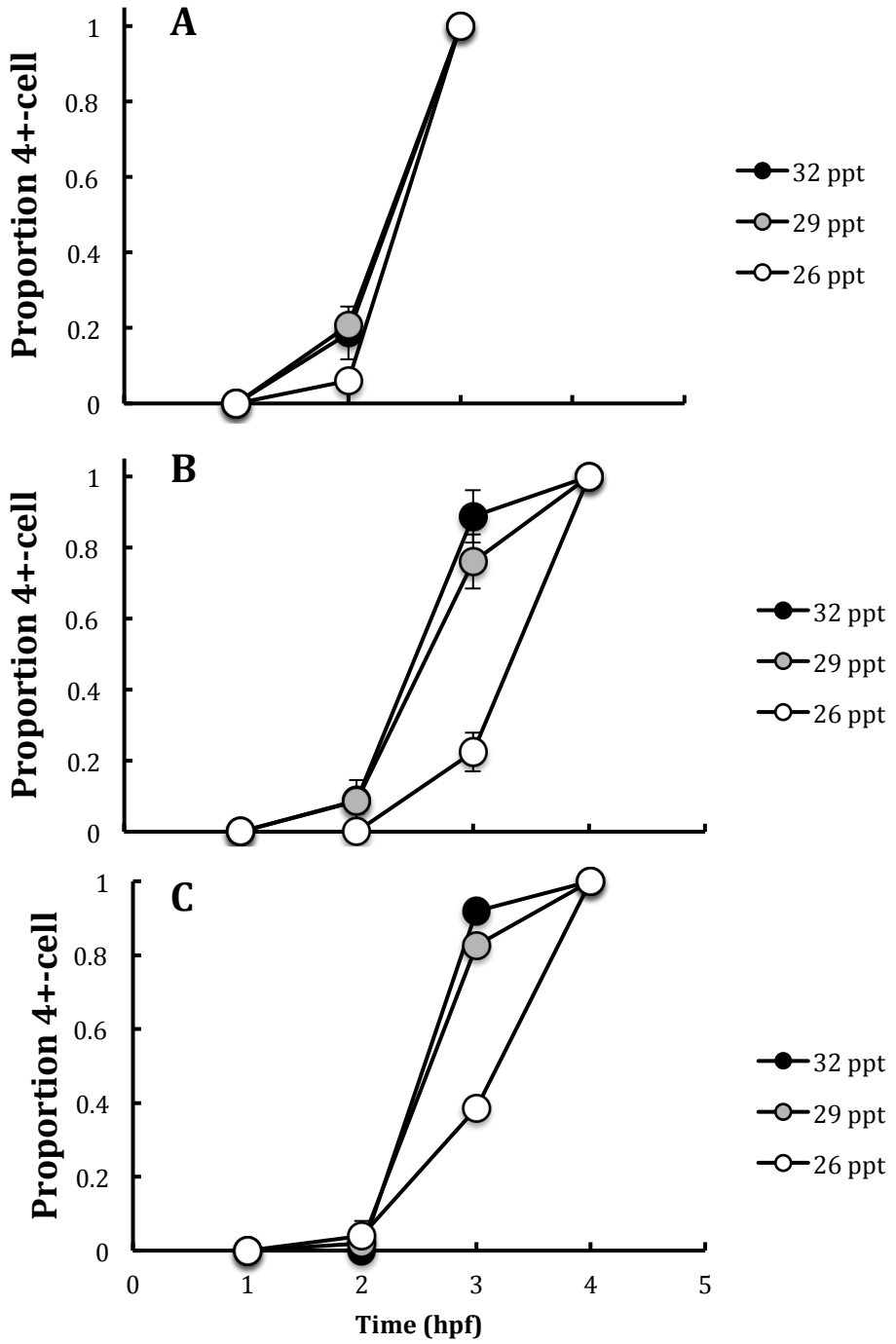


Figure 31: Cleavage to the 4+ cell stage in embryos of *D. excentricus* exposed to ambient water conditions (12-15°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$).

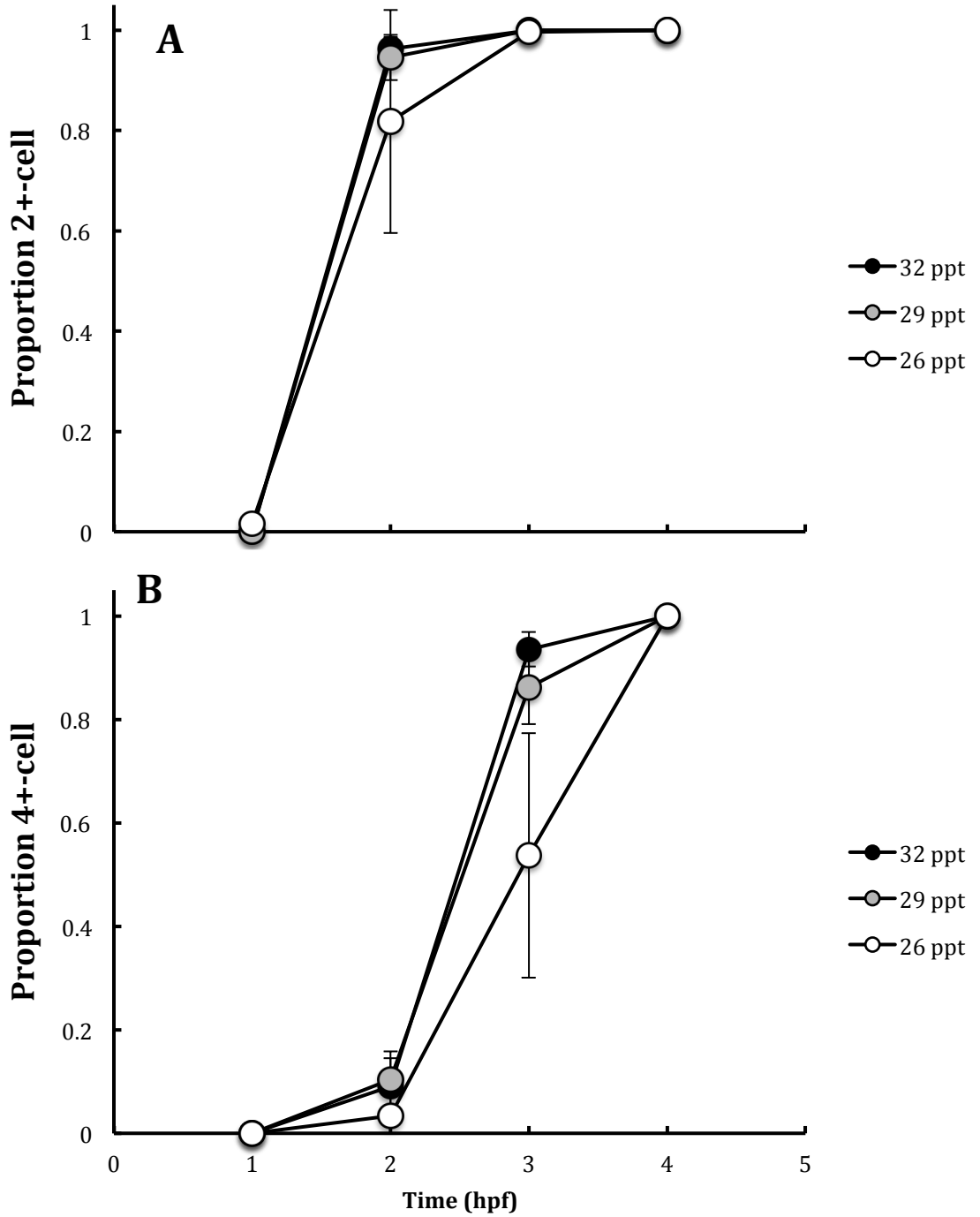


Figure 32: Cleavage to the 2+ cell (A) and 4+ cell (B) stage in embryos of *D. excentricus* exposed to ambient water conditions (12-15°C). Each point represents the mean ± standard error for three replicate trials. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$).

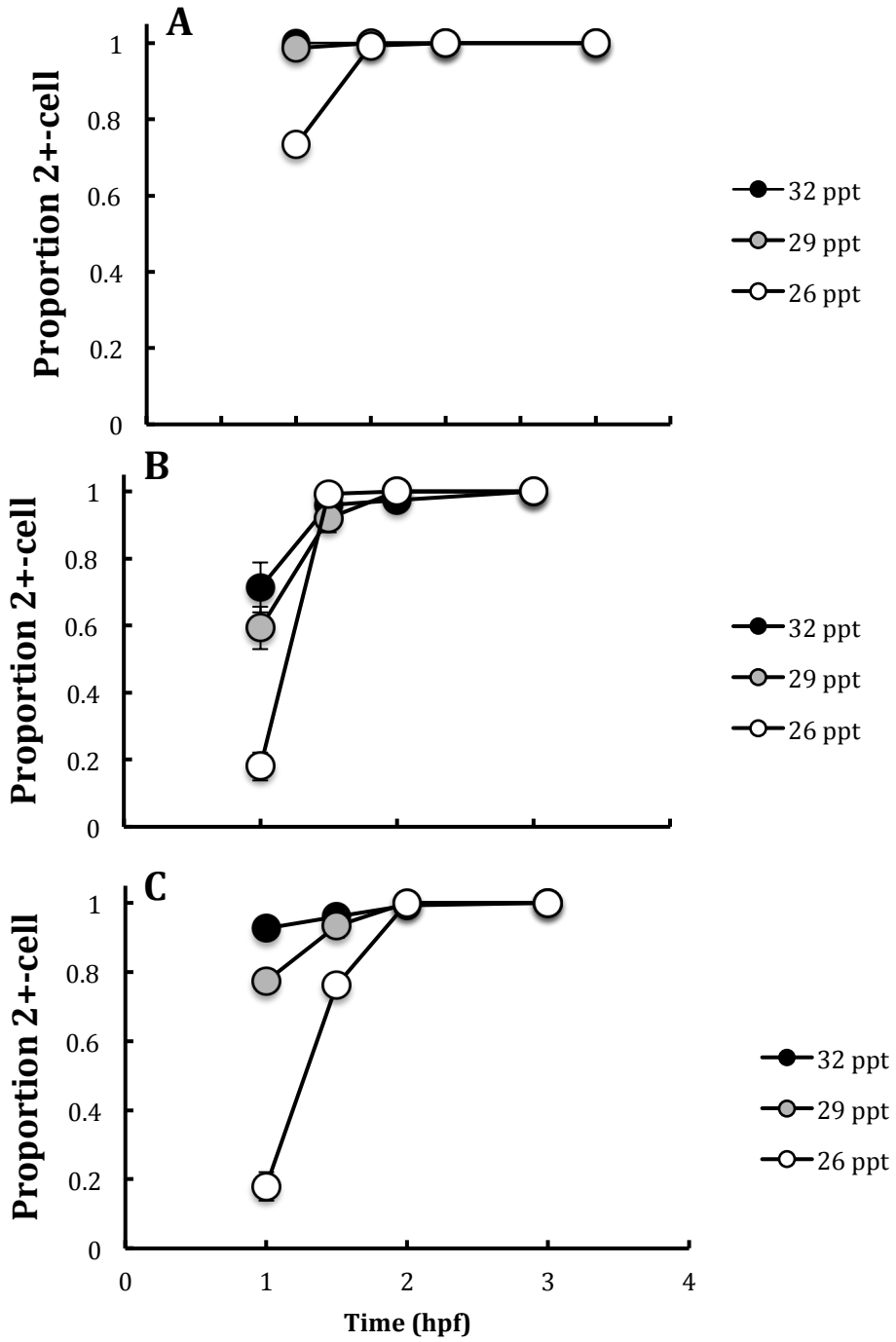


Figure 33: Cleavage to the 2+ cell stage in embryos of *D. excentricus* exposed to heated water conditions (19-23°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$). However, there seems to be a slight lag effect between embryos exposed to reduced salinities (29 and 26 ppt) and full strength seawater (32 ppt).

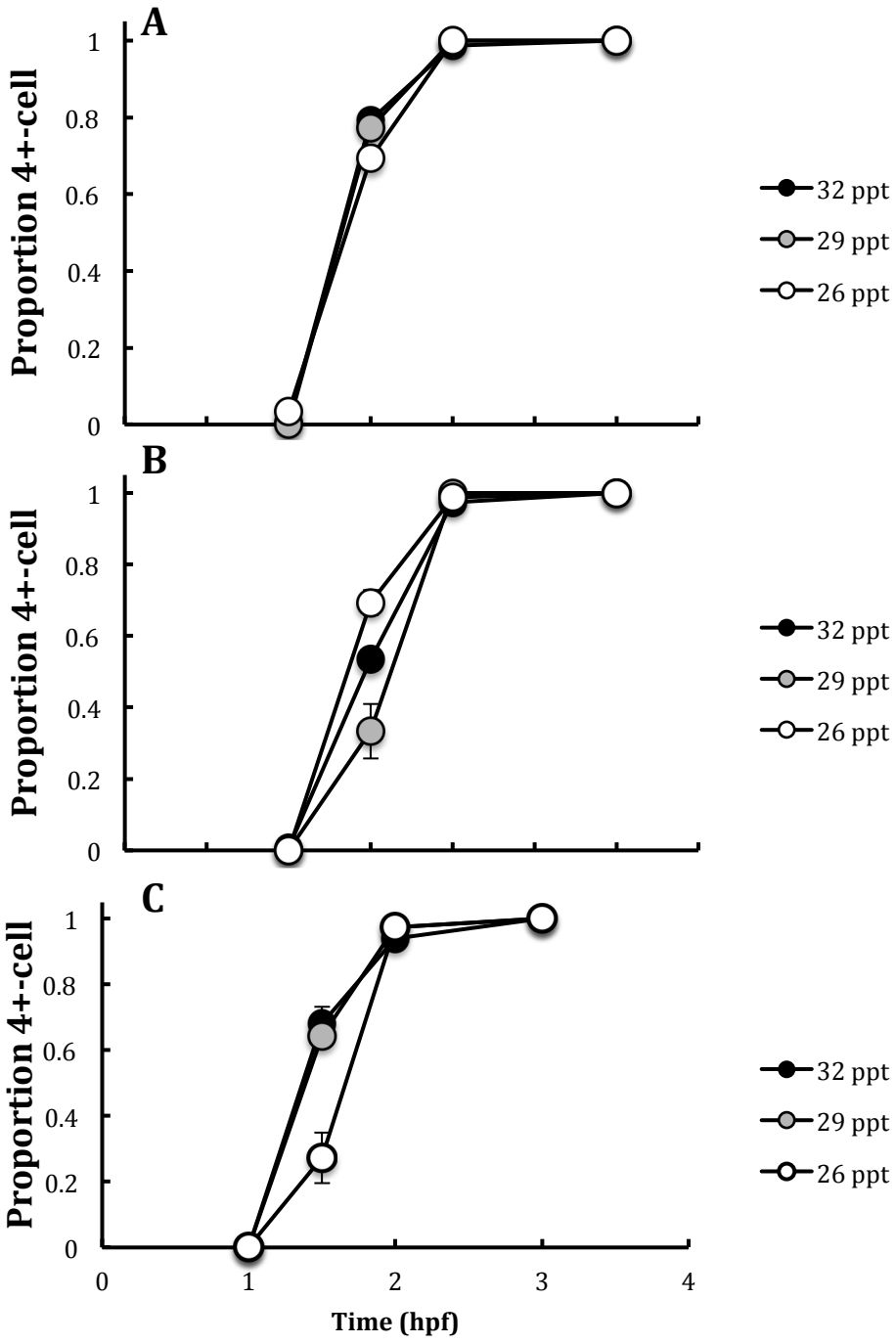


Figure 34: Cleavage to the 4+ cell stage in embryos of *D. excentricus* exposed to heated water conditions (19-23°C). Each graph represents a replicate parental pair (A-C). Each point represents the mean ± standard error for three replicate bowls. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$). The lag previously seen in cleavage to the 2+ cell stage does not seem to persist into cleavage to the 4+ cell stage.

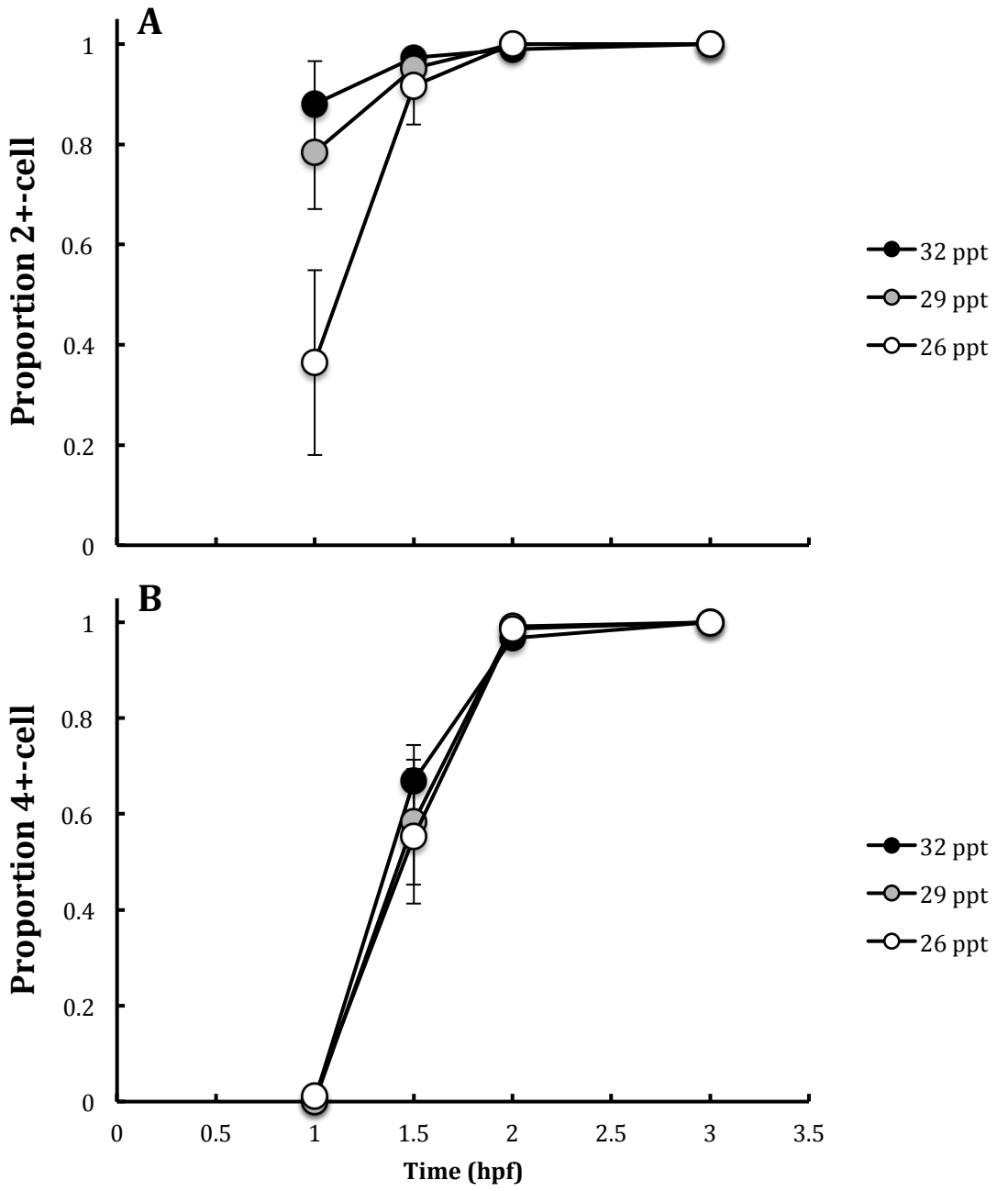


Figure 35: Cleavage to the 2+ cell (A) and 4+ cell (B) stage in embryos of *D. excentricus* exposed to heated water conditions (19-23°C). Each point represents the mean ± standard error for three replicate trials. Black circles indicate embryos exposed to 32 ppt seawater, grey indicates exposure to 29 ppt seawater, and white circles correspond to embryos maintained in 26 ppt seawater. Trial, temperature, and salinity were all found to not have significant effects on cleavage ($p > 0.050$), and time was found to have a significant effect ($p < 0.050$). A lag-like trend is observed in cleavage to the 2+ cell stage (A), but does not persist into the 4+ cell stage (B).

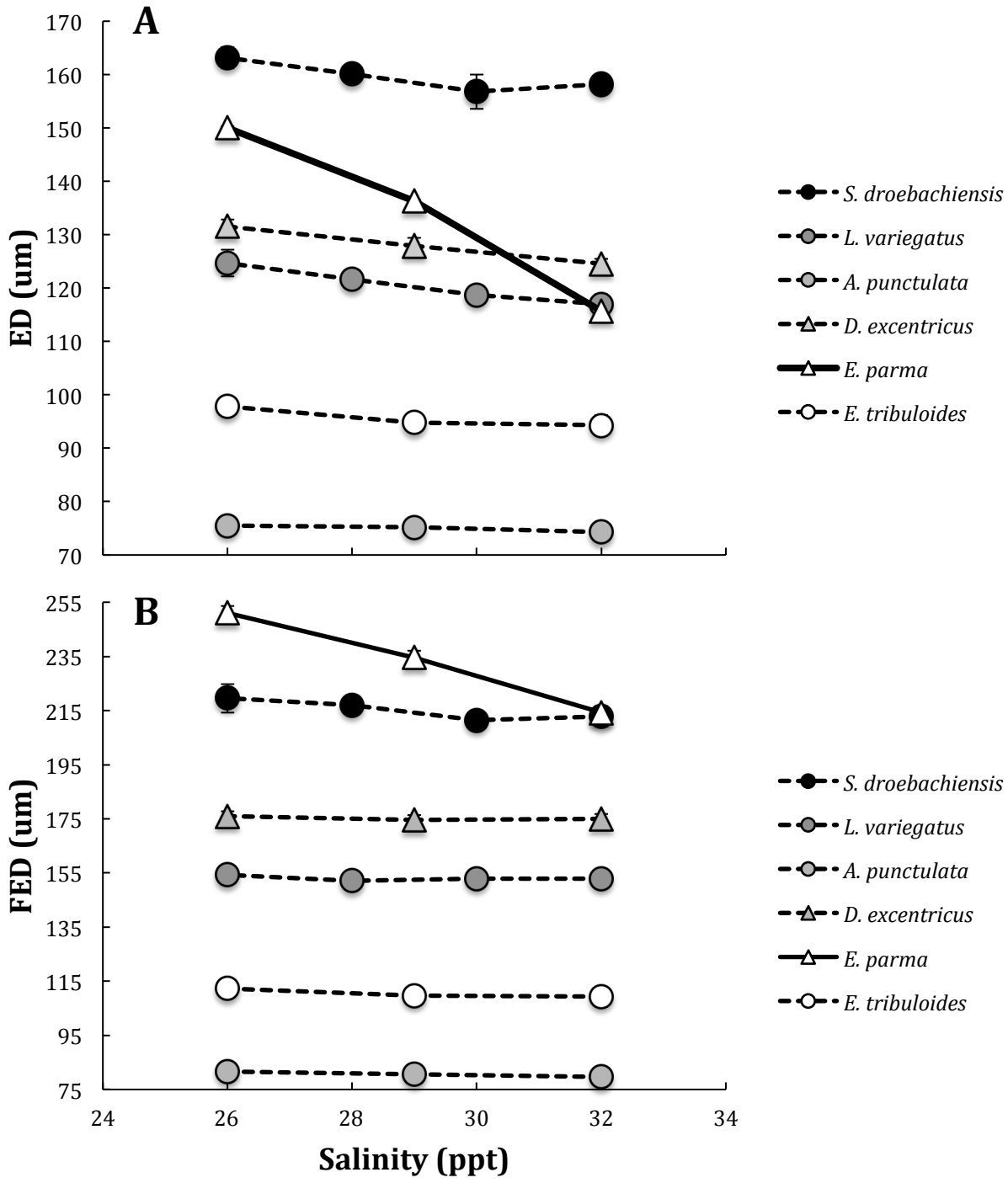


Figure 36: ED (A) and FED1 (B) measurement comparisons across all species studied. Each point is the mean ± standard error for three experimental trials for each species. The trend line for *E. parma* is bolded in both graphs to highlight the more drastic response embryos had to identical salinity exposures, whereas other species did not respond as strongly.

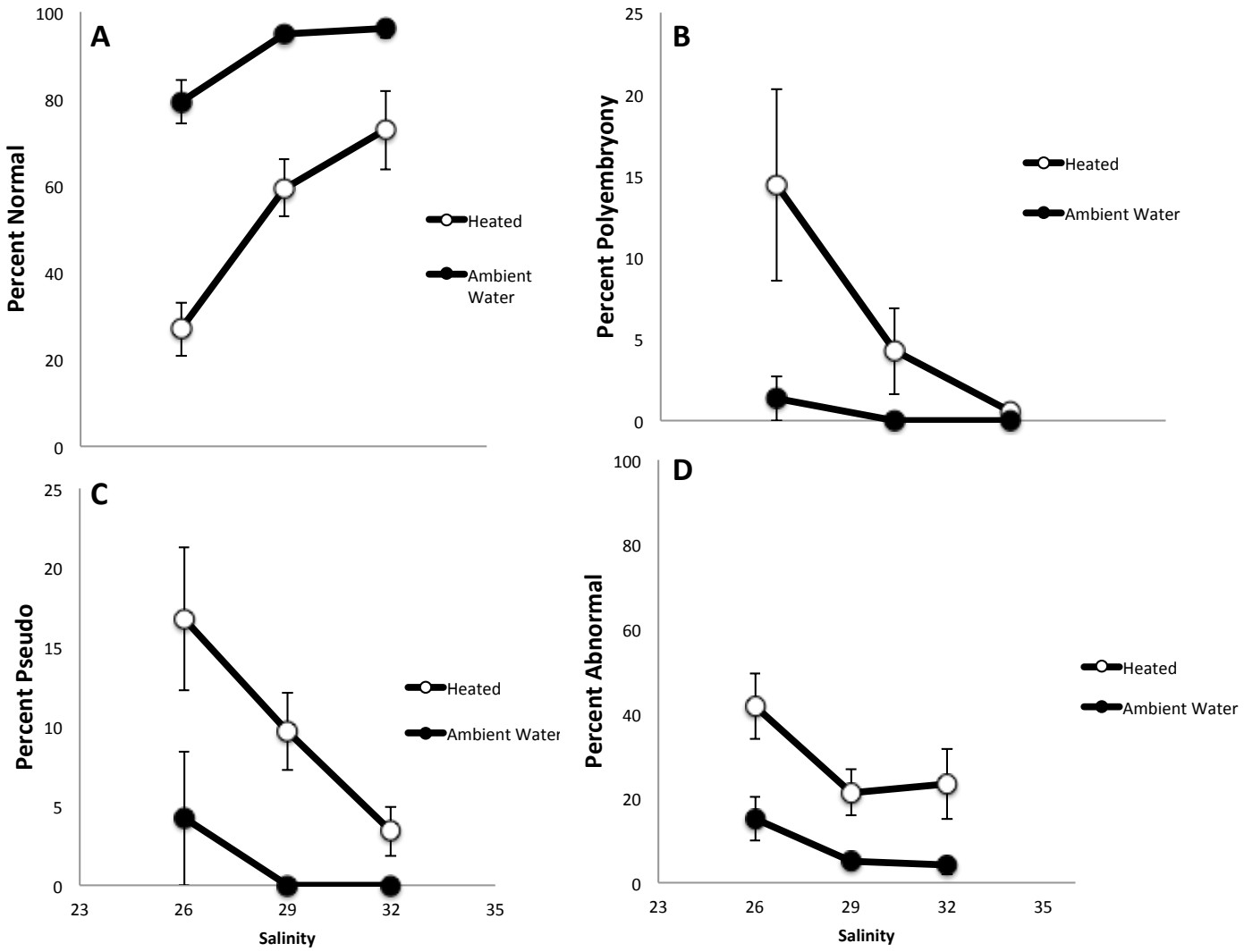


Figure 37: Percent normal (A), polyembryonic (B), pseudo-polyembryonic (C), and abnormal development (D) in embryos of *D. excentricus*. Each point represents the mean ± standard error for three replicate parental pairs. Black circles indicate embryos exposed to ambient seawater temperatures (12-15°C) and white circles correspond to embryos maintained heated water temperatures (19-23°C). Salinity, temperature, and their interaction were all found to not have significant effects on normal (A), polyembryonic (B), and pseudo-polyembryonic (C) development $p < 0.050$), but they were not significant predictors of abnormal (D) development ($p > 0.050$). Note change in y-axis scale between figures 37 A and D, and 37 B and C.

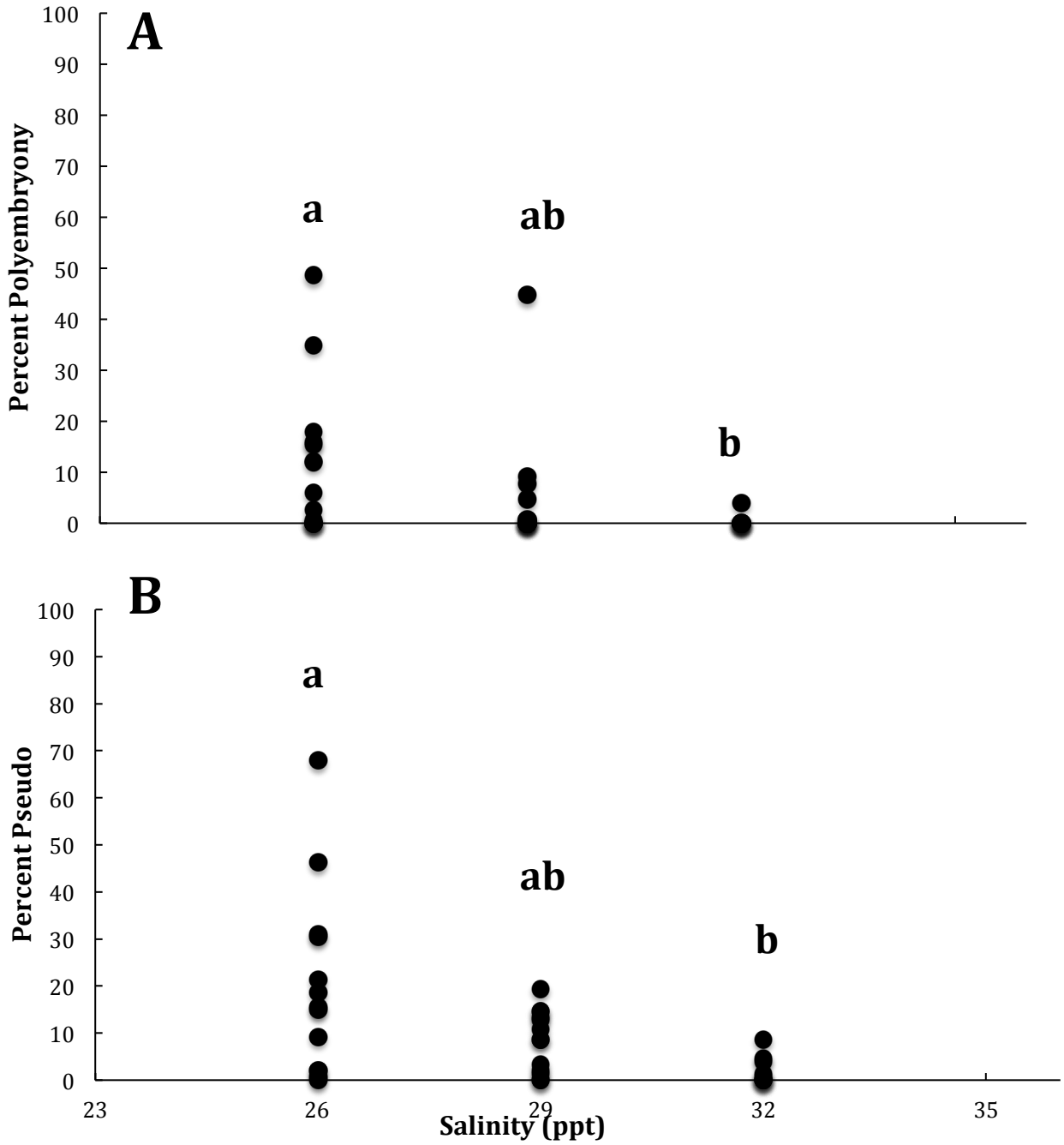


Figure 38: Variation in polyembryonic (A) and pseudo-polyembryonic (B) development across several parental pairs. Each point represents means from three replicate bowls. Lowercase letters indicate results of post-hoc, Bonferroni pairwise comparisons across salinities. For both poly and pseudo-polyembryonic development 29 ppt was not significantly different from either 26 ppt or 32 ppt salinity treatments ($p > 0.050$), but 26 and 32 ppt treatments were significantly different from one another ($p < 0.050$).

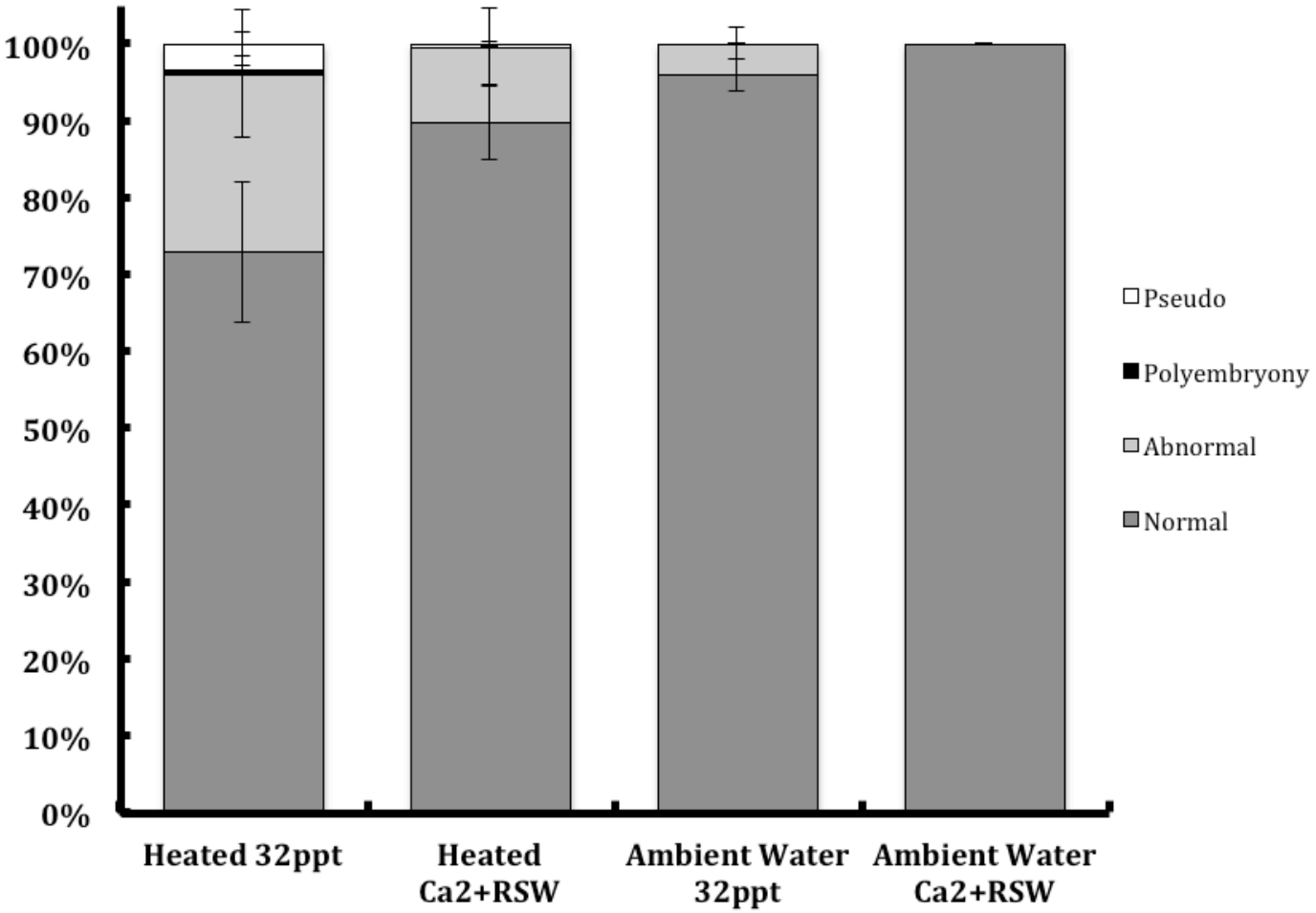


Figure 39: Proportions of normal development (dark grey), polyembryonic development (black), pseudo-polyembryonic development (white), and abnormal development (light grey) in embryos of *D. excentricus* exposed to both 32 ppt and Calcium reduced seawater at ambient and heated water temperatures. Bars represent means ± standard error from three replicate experimental trials. Calcium reduced seawater does not seem to increase the proportion of polyembryony in embryos. Embryos exposed to calcium reduced seawater have exhibited greater proportions of normal development, and lower proportions of abnormal development in comparison to embryos exposed to full-strength seawater.

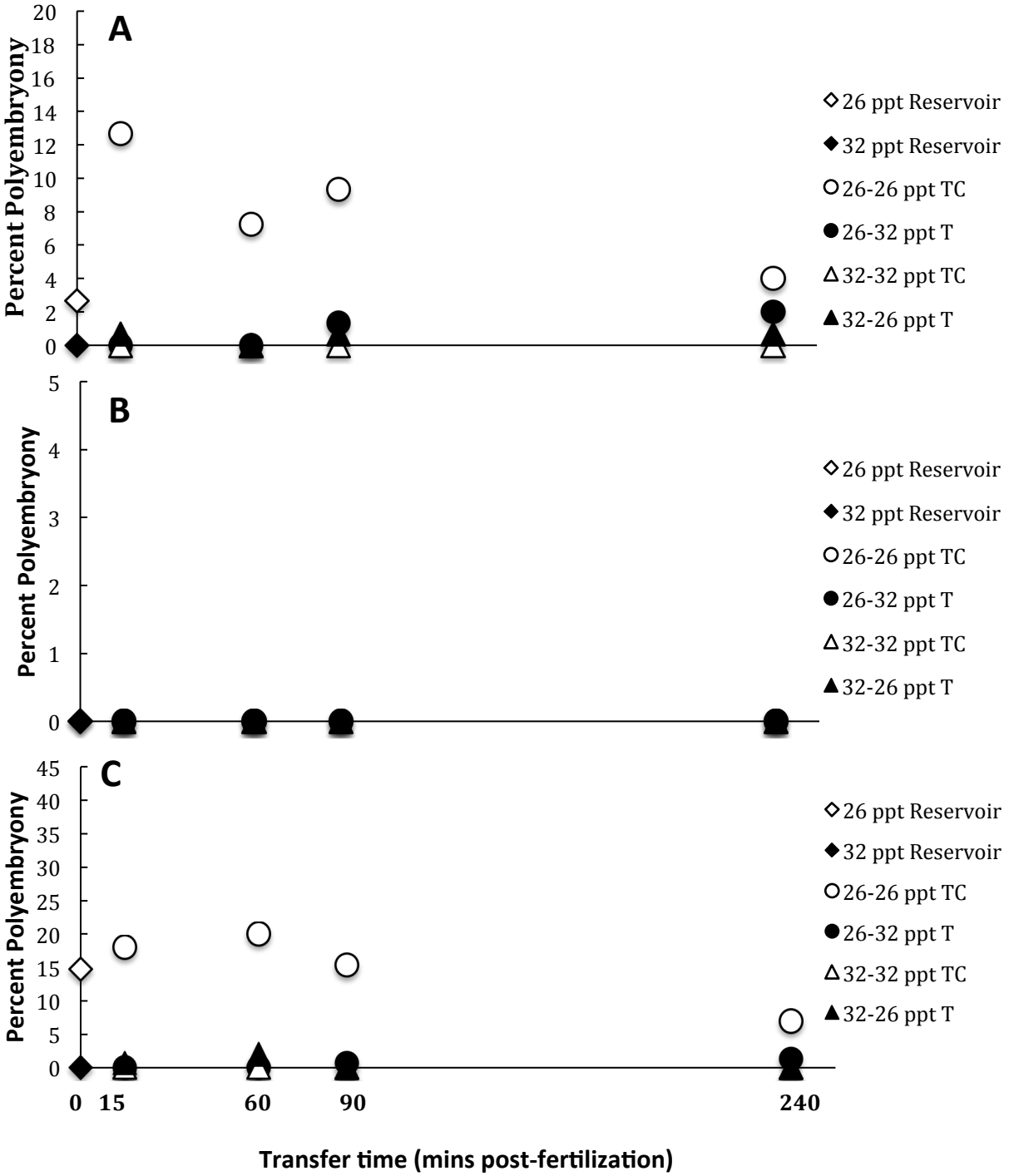


Figure 40: Percent polyembryony in reservoir bowls (white and black diamonds), transfer control (TC; white circles and triangles), and transfer treatments (black circles and triangles). Each graph represents a parental pair (A-C). Points represent means ± standard error from three replicate bowls. Shifts in percent polyembryony were not really observed across treatments. Note changes in y-axis scales across graphs.

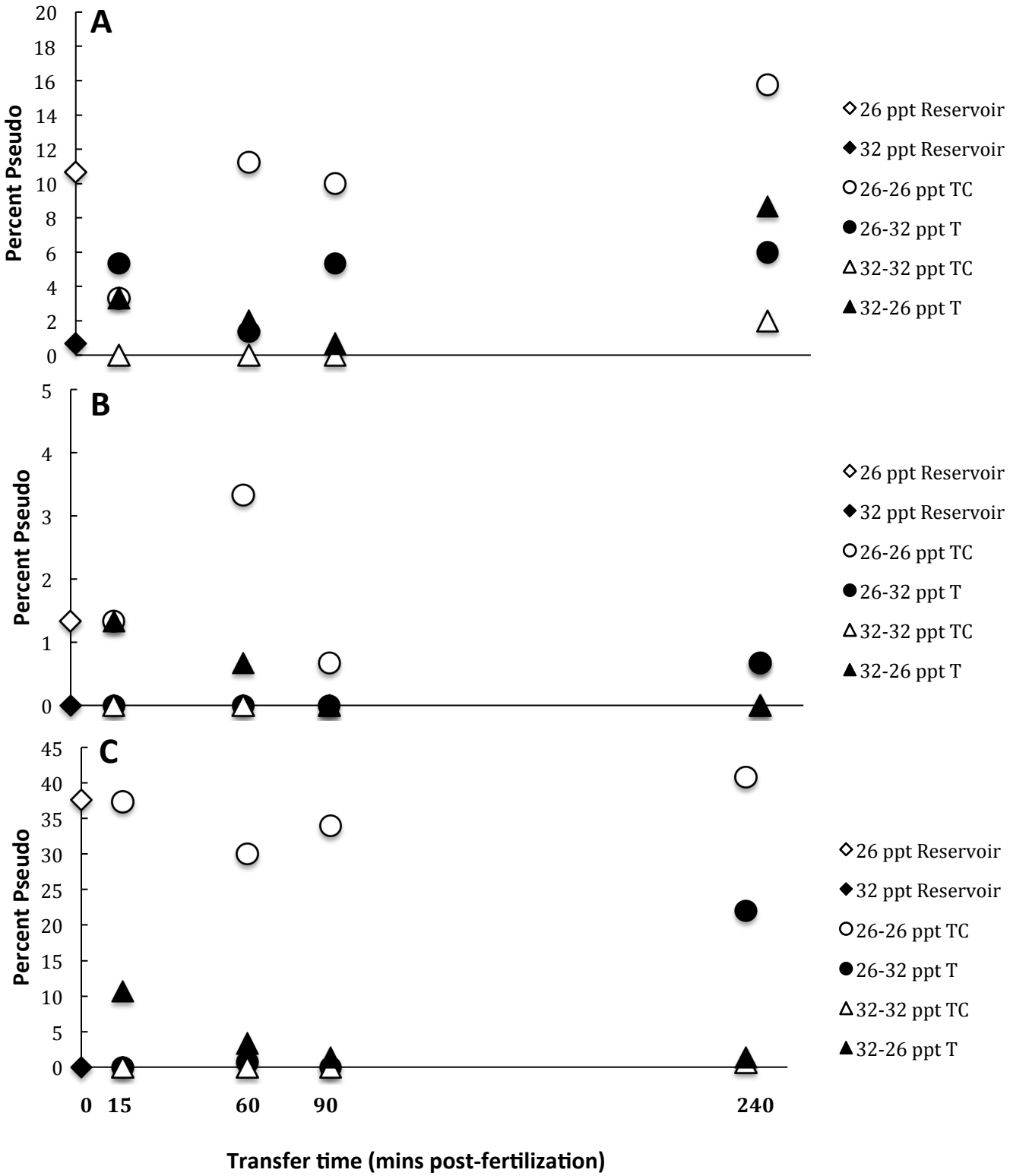


Figure 41: Percent pseudo-polyembryony in reservoir bowls (white and black diamonds), transfer control bowls (TC; white circles and triangles), and transfer bowls (black circles and triangles). Each graph represents a parental pair (A-C). Points represent means ± standard error from three replicate bowls. Shifts in percent pseudo-polyembryony were observed across treatments even in treatments that were transferred 240 mpf, which is about 2-3 hours prior to hatching. Note changes in y-axis scales across graphs.

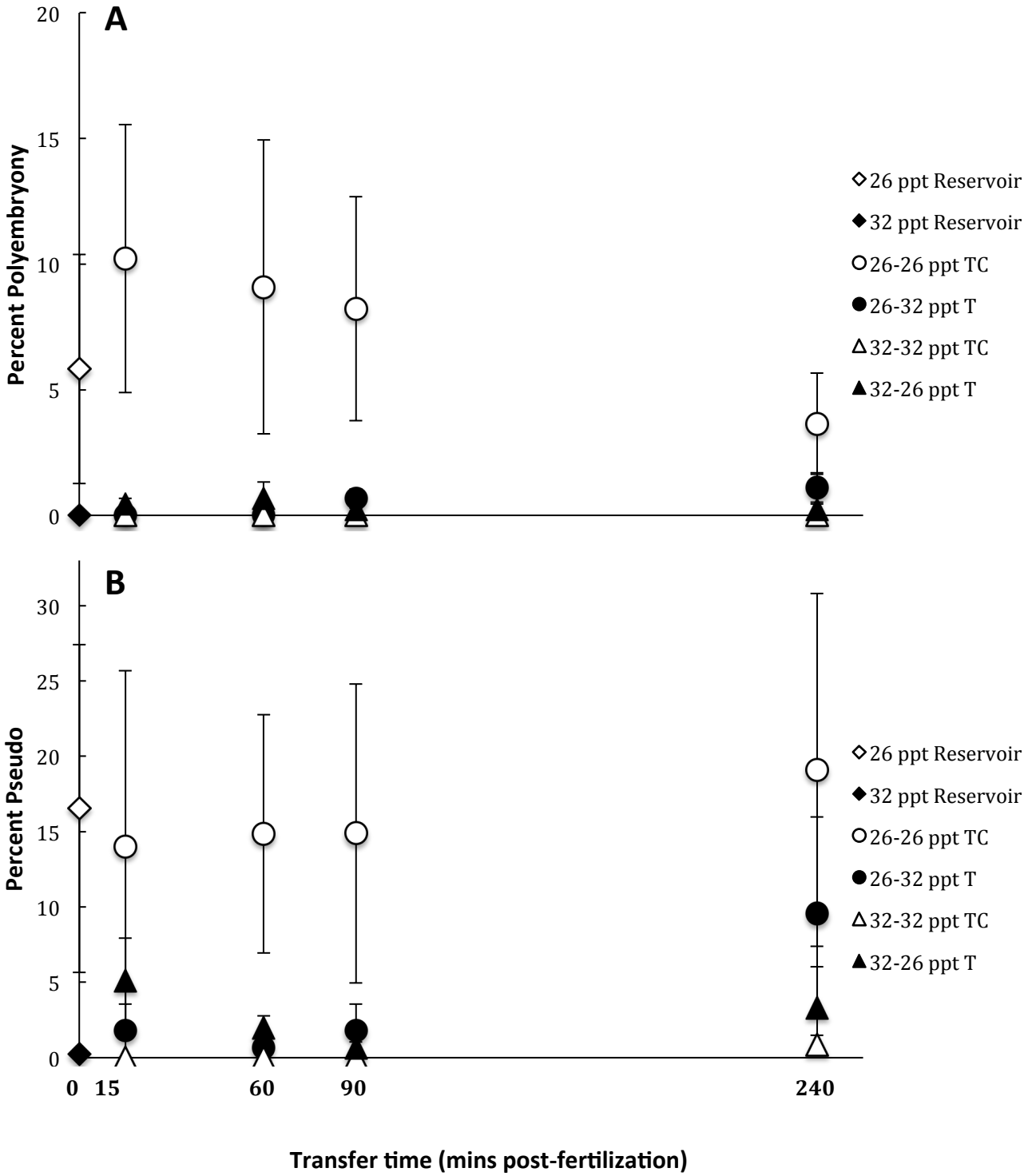


Figure 42: Percent polyembryony (A) and pseudo-polyembryony (B) in reservoir bowls (white and black diamonds), transfer control bowls (TC; white circles and triangles), and transfer bowls (black circles and triangles). Points represent means ± standard error from three replicate parental pairs. Patterns observed in either developmental morphology from individual experimental graphs, described in figures 41 and 42, persisted into these overall graphs. Note changes in y-axis scales between graphs.

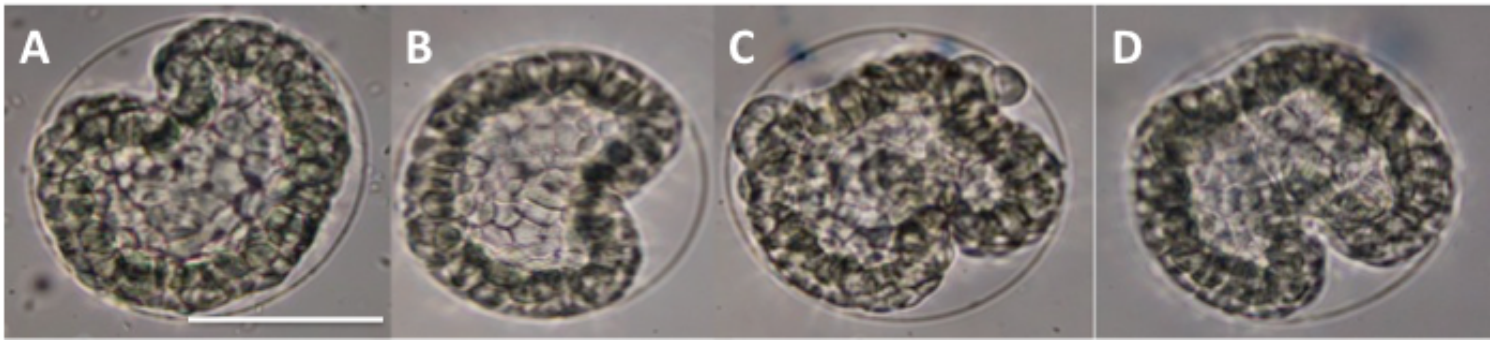


Figure 43: Various types of *D. excentricus* pseudo-twin embryos. Figures A and B are more representative of class 2 pseudo-twins, whereas figures C and D more closely resemble class 3 pseudo-twins. Scale bar represents 100 μm.

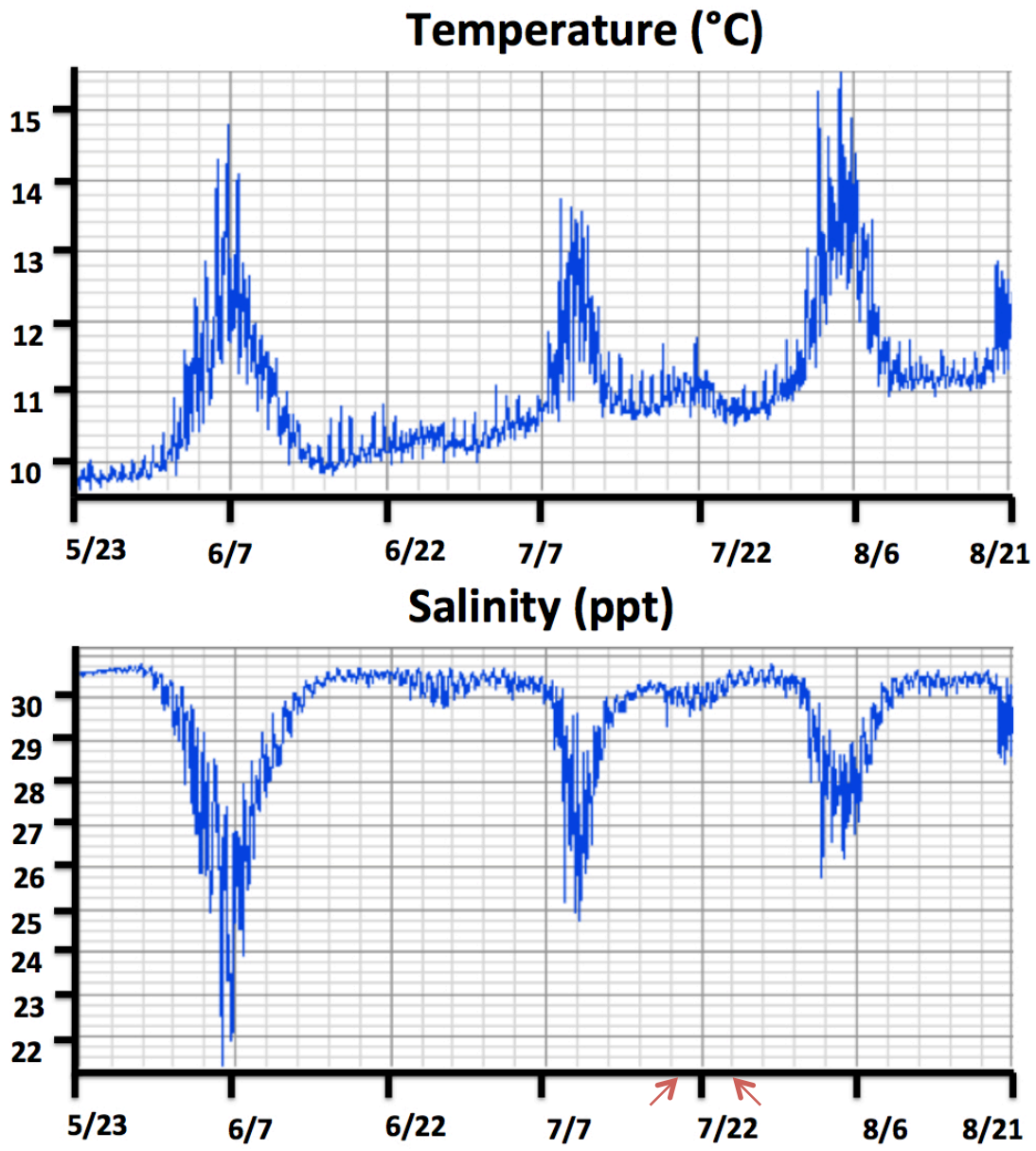


Figure 44: Salinity and temperature data obtained from the FHL underwater monitoring station at Cantilever Point, located 1.7 meters below MLLW (indicated by red dot on Figure 4), courtesy of Dr. Emily Carrington. Increases in temperature and salinity decreases are shown for the spawning period of sand dollars and are directly correlated with periodic freshwater input from the Fraser River. The maximum temperature observed was about 15.5 °C and the minimum salinities observed were about 21.3 ppt for this time period. All data was collected in 2014.

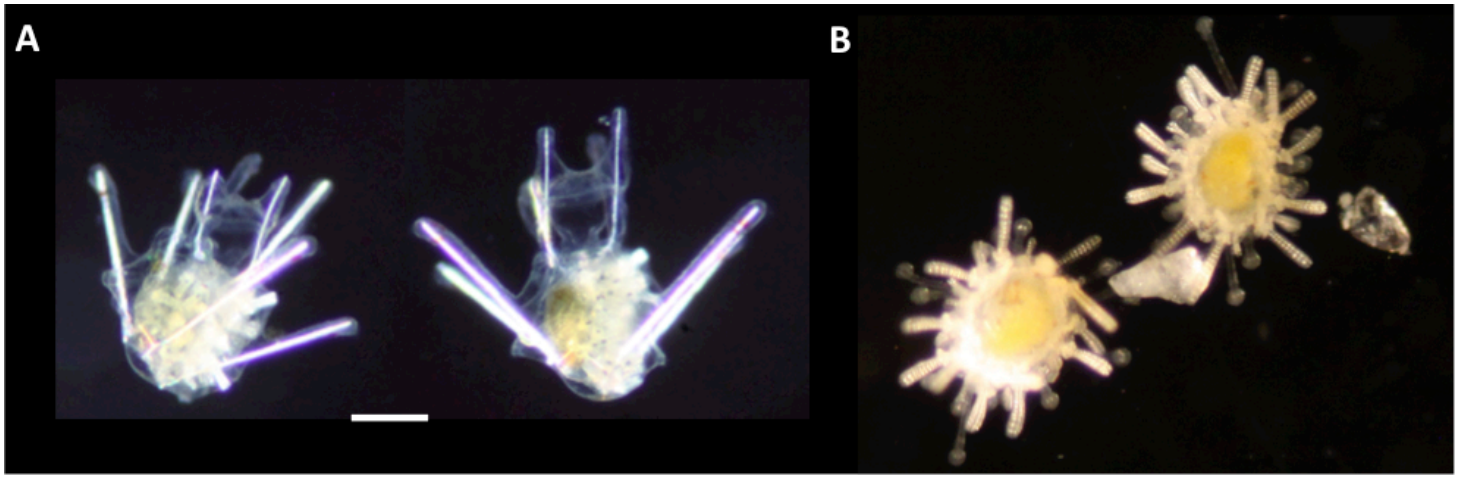


Figure 45: A set of twins, late in the larval development period (A) as indicated by the presence of the juvenile rudiment. Larval skeletal rods are fluoresced via a microscopic lens polarizing filter to aid in their visualization. A set of twins that have settled and metamorphosed into the juvenile stage (B). Scale bar represents 100 μm.

Tables

Table 1: Summary of the experimental treatments used on embryos of the various species described.

Species	Salinity Treatments	Temperature Treatments
S. droebachiensis	32, 30, 28, and 26 ppt	Ambient (8°C)
A. punctulata	32, 29, and 26 ppt	Ambient (23°C)
L. variegatus	32, 30, 28, and 26 ppt	Ambient (21-24°C)
E. tribuloides	32, 29, and 26 ppt	Ambient (24-26°C)
E. parma	32, 29, and 26 ppt	Ambient (12-13°C)
D. excentricus	32, 29, 26 ppt, and Ca^{2+}RSW	Ambient (12-15°C) Heated (19-23°C)

Table 2 *S. droebachiensis* hatching: Binomial regression analyses were conducted on hatching data collected from each experimental trial. Salinity and age (hours post-fertilization – hpf) were all found to have significant effects on hatching.

Predictor	**β**	**SE β**	**Wald's X^2**	**df**	**p**	**e^{β} (odds ratio)**
Trial 1:						
Age	-8.850	0.428	427.568	1	< 0.001	< 0.001
Salinity	-10.675	5.17	425.717	1	< 0.001	< 0.001
Age x Salinity	0.364	0.016	488.616	1	< 0.001	1.439
Trial 2:						
Age	-17.656	0.929	361.317	1	< 0.001	< 0.001
Salinity	-22.099	1.151	368.599	1	< 0.001	< 0.001
Age x Salinity	0.718	0.036	329.251	1	< 0.001	2.049
Trial 3:						
Age	-4.788	0.404	140.331	1	< 0.001	0.008
Salinity	-5.892	0.489	145.277	1	< 0.001	0.003
Age x Salinity	0.220	0.015	204.921	1	< 0.001	1.245

Table 3: *S. droebachiensis* ED and FED: ANOVAs were conducted on means calculated from all three experimental trials. Salinity alone was found to have a significant effect on ED and FED. However, time and its interaction with salinity was found to have a non-significant ($p > 0.050$) effect on FED indicating that there was little/no difference between the two measurements.

Predictor	Numerator df	Denominator df	F-value	p
ED				
Salinity	3	30	24.381	< 0.001
FED				
Salinity	3	62	15.815	< 0.001
Time	1	62	0.911	0.343
Salinity X Time	3	62	0.319	0.811

Table 4 *A. punctulata* hatching: Binomial regression analyses were conducted on hatching data from each experimental trial. Salinity and age (hpf) were all found to have significant effects on hatching, except for in the third trial.

Predictor	β	SE β	Wald's X^2	df	p	e^{β} (odds ratio)
Trial 1:						
Age	-6.659	2.224	8.968	1	0.003	0.001
Salinity	-1.859	0.590	9.945	1	0.002	0.156
Age x Salinity	0.309	0.077	16.331	1	< 0.001	1.363
Trial 2:						
Age	-6.066	2.535	5.727	1	0.017	0.002
Salinity	-2.042	0.570	12.831	1	< 0.001	0.130
Age x Salinity	0.317	0.093	11.673	1	0.001	1.373
Trial 3:						
Age	14.914	10990.535	< 0.001	1	0.999	3000417.26
Salinity	-0.791	2647.212	< 0.001	1	1.000	0.454
Age x Salinity	0.100	378.173	< 0.001	1	1.000	1.105

Table 5 *A. punctulata* ED and FED: ANOVAs were conducted on means calculated from all three experimental trials. Salinity was found to have a significant effect on ED. Salinity and time were both found to have significant effects on FED ($p < 0.050$), whereas their interaction was found to have a non-significant ($p > 0.050$) effect on FED.

Predictor	Numerator df	Denominator df	F-value	p
ED				
Salinity	2	22	5.056	0.016
FED				
Salinity	2	46	15.390	< 0.001
Time	1	46	8.129	0.007
Salinity X Time	2	46	2.937	0.063

Table 6 *L. variegatus* hatching: Binomial regression analyses were conducted on hatching data from each experimental trial. Salinity and age (hpf) were all found to have significant effects on hatching.

Predictor	β	SE β	Wald's X^2	df	p	e^{β} (odds ratio)
Trial 1:						
Age	-10.698	1.618	43.719	1	< 0.001	< 0.001
Salinity	-3.072	0.670	21.008	1	< 0.001	0.046
Age x Salinity	0.413	0.057	52.532	1	< 0.001	1.511
Trial 2:						
Age	-8.792	1.994	19.448	1	< 0.001	< 0.001
Salinity	-2.421	0.701	11.936	1	0.001	0.089
Age x Salinity	0.334	0.066	25.679	1	< 0.001	1.396
Trial 3:						
Age	-2.851	0.692	16.973	1	< 0.001	0.058
Salinity	-1.032	0.287	12.958	1	< 0.001	0.356
Age x Salinity	0.137	0.024	33.339	1	< 0.001	1.147

Table 7 *L. variegatus* ED and FED: ANOVAs were conducted on means calculated from all three experimental trials. Salinity was found to have a significant effect on ED. Time was the only predictor which had a significant effect on FED ($p < 0.050$). Salinity and its interaction with time, were both not found to have significant effects on FED ($p > 0.050$).

Predictor	Numerator df	Denominator df	F-value	p
ED				
Salinity	3	30	50.685	< 0.001
FED				
Salinity	3	54	2.469	0.072
Time	1	54	25.548	< 0.001
Salinity X Time	2	54	0.107	0.898

Table 8 *E. tribuloides* ED and FED: An ANOVA was conducted on means calculated from the sole experimental trial for this species. Salinity was not found to have a significant effect on ED ($p > 0.050$). Salinity and time independently had a significant effect on FED ($p < 0.050$), however their interaction was not significant ($p > 0.050$).

Predictor	Numerator df	Denominator df	F-value	p
ED				
Salinity	2	6	1.955	0.222
FED				
Salinity	2	12	5.357	0.022
Time	1	12	10.637	0.007
Salinity X Time	2	12	0.236	0.793

Table 9: *E. parma* hatching: A univariate ANOVA was conducted on hatching data generated from the single transfer experiment conducted on embryos of *E. parma*. Only hatching data from the initial fertilization, non-transfer bowls was used in this analysis. Salinity was coded as a fixed effect and replicate bowl was coded as a random effect to account for any differences among bowls. Salinity was found to have a significant effect ($p < 0.050$) on hatching whereas bowl was not found to be a significant predictor of hatching (($p > 0.050$).

Predictor	Type III Sum of Squares	df	Mean Square	F-value	p
Salinity					
Hypothesis	172.980	2	12.847	78.761	< 0.001
Error	78.233	445	0.176		
Bowl					
Hypothesis	0.093	2	0.047	0.265	0.767
Error	78.233	445	0.176		

Table 10: *E. parma* ED and FED: An ANOVA was conducted on means calculated from the sole experimental trial for this species. Salinity was found to have a significant effect on ED ($p < 0.050$). Salinity, time, and their interaction were all shown to be significant predictors of hatching ($p < 0.050$).

Predictor	Type III Sum of Squares	df	Mean Square	F-value	p
ED					
Salinity	1792.667	2	896.333	52.045	< 0.001
FED					
Salinity	2478.778	2	1239.389	79.391	< 0.001
Time	522.722	1	522.722	33.484	< 0.001
Salinity X Time	192.111	2	96.056	6.153	0.014

Table 11: Summary of only p-values generated from binomial regression analyses conducted on each replicate hatching trial on embryos of *D. excentricus*. P-values for each predictor (temperature, age, and salinity) and their various interactions are listed.

Trial	Temperature	Age (hpf)	Salinity	Age X Temp.	Sal. X Temp.	Age X Sal.	Age X Sal. X Temp.
1 Full Model	0.019	0.457	0.554	0.052	0.009	0.667	0.173
1 Reduced Model	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	--	< 0.001
2 Full Model	0.18	0.711	0.88	0.13	0.46	0.063	0.958
2 Reduced Model	0.768	< 0.001	< 0.001	--	< 0.001	< 0.001	--
3 Full Model	< 0.001	0.003	< 0.001	< 0.001	< 0.001	0.09	< 0.001

Table 12 *D. excentricus* hatching: Binomial regression analyses were conducted on hatching data from each experimental trial. Salinity and age (hpf) were all found to have significant effects on hatching. Temperature was found to be a significant predictor of hatching in trials 1 and 3, but not trial 2. P-values from reduced regression models only are reported below. See table 11 for p-values from full and reduced models.

Predictor	β	SE β	Wald's X^2	df	p	e^{β} (odds ratio)
Trial 1:						
Temperature	61.171	9.562	40.923	1	< 0.001	$3.682 * 10^{26}$
Age	4.883	0.367	176.877	1	< 0.001	132.006
Salinity	1.363	0.229	35.527	1	< 0.001	3.907
Age X Temp.	-5.226	0.431	147.362	1	< 0.001	0.005
Salinity X Temp.	-2.379	0.351	45.992	1	< 0.001	0.093
Age X Sal. X Temp	0.139	0.016	79.885	1	< 0.001	1.149
Trial 2:						
Temperature	-0.328	4.420	0.006	1	0.941	0.720
Age	-3.507	0.595	34.720	1	< 0.001	0.030
Salinity	-0.744	0.080	87.009	1	< 0.001	0.475
Salinity X Temp.	-0.599	0.157	14.589	1	< 0.001	0.549
Age X Salinity	0.207	0.022	90.485	1	< 0.001	1.230
Trial 3:						
Temperature	125.376	21.549	33.852	1	< 0.001	$2.820 * 10^{54}$
Age	12.497	4.235	8.707	1	0.003	267654.344
Salinity	4.693	1.292	13.201	1	< 0.001	109.127
Age X Temp.	-9.799	2.228	19.336	1	< 0.001	< 0.001
Salinity X Temp.	-4.576	0.773	35.042	1	< 0.001	0.010
Age X Salinity	-0.252	0.149	2.870	1	0.090	0.777
Age X Sal. X Temp.	0.288	0.079	13.292	1	< 0.001	1.334

Table 13: Summary of p-values generated from fitting ED and FED data to two tests of normality.

Transformation	Kolmogorov-Smirnov **p**	Shapiro-Wilk **p**
FED	0.014	0.001
FED_sqrt transformation	0.013	0.002
FED_Log_{10} transformation	0.2	0.269
FED_LN transformation	0.013	0.003

Table 14 *D. excentricus* ED and FED: An ANOVA was conducted on Log_{10} transformed ED and FED1 means calculated from all three experimental trials. Salinity was found to have a significant effect on ED. Whereas time was the only predictor which had a significant effect on FED ($p < 0.050$). Salinity and temperature, were both not found to have significant effects on FED ($p > 0.050$). Interactions among the three variables are left out due to $p > 0.250$.

Predictor	Numerator df	Denominator df	F-value	p
ED				
Salinity	2	48	130.903	< 0.001
Temperature	1	48	1.052	0.310
FED log_{10} transformation				
Temperature	1	80.139	1.892	0.172
Salinity	2	80.093	1.198	0.307
Time	1	80.482	13.239	< 0.001

Table 15: Summary of polyembryonic FED2 (Pemb FED2) and non-polyembryonic FED2 data found across experimental trials in differing salinity treatments and bowl replicates. Differences between polyembryonic FED2 and non-polyembryonic FED2 are also shown.

Treatment	FED2	Pemb FED2	Difference Pemb FED2 - FED2
26_Heated_i	172.025	173.9166667	1.891666667
29_Heated_ii	187.025	179.9166667	-7.108333333
26_Heated_ii	184.2666667	183.9833333	-0.283333333
29_Heated_iii	184.0166667	174	-10.01666667
26_Heated_iii	171	176.7666667	5.766666667
26_Ambient_ii	182.8333333	184	1.166666667

Table 16 *D. excentricus* cleavage: Means of data from three replicate trials were analyzed using a non-parametric independent sample Kruskal-Wallis test. Trial, temperature, and salinity were all found to be non-significant predictors of hatching to either the 2 or 4 cell stage, whereas time was the only significant predictor of cleavage.

Predictor	p
2+ Cells	
Trial	0.191
Temperature	0.290
Salinity	0.833
Time	< 0.001
4+ Cells	
Trial	0.792
Temperature	0.088
Salinity	0.963
Time	< 0.001

Table 17 Cross-Species ANOVA: Hatching data from embryos generated from three replicate parental pairs was analyzed using an ANOVA. Both species and salinity were found to have a significant ($p < 0.050$) effect on hatching of embryos, however, the interaction between salinity and species was not found to be significant ($p > 0.050$).

Variable	Numerator df	Denominator df	F-value	p
Species	4	14	35.133	.000
Salinity	3	14	3.719	.037
Species X Salinity	4	14	1.551	.242

Table 18A Normal development: ANOVA was conducted on means generated from three experimental pairs on normal development in embryos of *D. excentricus*. Salinity, temperature, and their interaction were found to be significant ($p < 0.050$) predictors of normal development of embryos in this species.

Variable	Numerator df	Denominator df	F-value	p
Temperature	1	10	33.149	.000
Salinity	2	10	47.559	.000
Temperature X Salinity	2	10	8.504	.007

Table 18B Polyembryonic development: ANOVA was conducted on means generated from three experimental pairs on polyembryonic development in embryos of *D. excentricus*. Salinity, temperature, and their interaction were found to be significant ($p < 0.050$) predictors of polyembryonic development of embryos in this species.

Variable	Numerator df	Denominator df	F-value	p
Temperature	1	10	15.121	.003
Salinity	2	10	14.396	.001
Temperature X Salinity	2	10	6.793	.014

Table 18C Pseudo-polyembryonic development: ANOVA was conducted on means generated from three experimental pairs on pseudo-polyembryonic development in embryos of *D. excentricus*. Salinity, temperature, and their interaction were found to be significant ($p < 0.050$) predictors of pseudo-polyembryonic development of embryos in this species.

Variable	Numerator df	Denominator df	F-value	p
Temperature	1	12	16.380	.002
Salinity	2	12	8.249	.006
Temperature X Salinity	2	12	3.300	.072

Table 18D Abnormal development: ANOVA was conducted on means generated from three experimental pairs on abnormal development in embryos of *D. excentricus*. Salinity, temperature, and their interaction were not found to be significant ($p > 0.050$) predictors of abnormal development of embryos in this species.

Variable	Numerator df	Denominator df	F-value	p
Temperature	1	10	.002	.962
Salinity	2	10	1.889	.201
Temperature X Salinity	2	10	.193	.827

Table 19 *D. excentricus* polyembryonic and pseudo-polyembryonic variation: ANOVAs were conducted on means calculated from replicate bowls for several trials trial for this species. Salinity and temperature were found to have significant effects on polyembryonic development ($p < 0.050$), whereas their interaction was not significant ($p > 0.050$). Salinity and temperature independently were found to be significant predictors of pseudo-polyembryonic development ($p < 0.050$), their interaction was excluded from the model as it yielded a $p > 0.250$.

Predictor	Numerator df	Denominator df	F-value	p
Polyembryony				
Temperature	1	38	4.932	0.032
Salinity	2	29	4.059	0.028
Temperature X Salinity	2	29	1.745	0.192
Pseudo-Polyembryony				
Temperature	1	44	7.190	0.006
Salinity	2	20	3.271	0.010

Table 20A: *D. excentricus* pseudo-polyembryonic development: Table summary of proportions of embryos from specified pseudo-twin classes that continued to produce morphologically normal larvae. Over 80 % of class 2 and 3 pseudo-twins produced normal larvae.

D. excentricus

Proportion Normal Class 1	**Percent**
0.8393	83.93%
Proportion Normal Class 2&3	**Percent**
0.8278	82.78%
Proportion Normal Class 4	**Percent**
0.2766	27.66%

Table 20B *E. tribuloides* pseudo-polyembryonic development: Table summary of proportions of embryos from specified pseudo-twin classes that continued to produce morphologically normal larvae. Over 90 % of class 2 and 3 pseudo-twins produced normal larvae.

E. tribuloides

Proportion Normal Class 2	**Percent**
0.9655	96.55%
Proportion Normal Class 3	**Percent**
0.9375	93.75%

Table 21A Field data collected on 18 July 2014

Location	Time	Depth (ft)	Salinity (ppt)	Temperature (°C)
N 48° 38.365' W 122° 52.652'	15:44	4	31.6	21.0
N 48° 38.365' W 122° 52.625'	15:47	4	31.2	20.2
Madrona Point	15:50	10	31.7	17.5
Middle of East Sound N 48° 38.593' W 122° 52.995'	16:04	40	32.3	15.0
Front of East Sound N 48° 37.007' W 122° 51.703'	16:12	95	32.5	13.2

Table 21B Field data collected on 25 July 2014

Location	Time	Salinity (ppt)	Temperature (°C)	pH
White Beach	09:51	32.4	14.5	8.17
Rosario	09:59	32.3	14.9	8.27
Madrona Point	10:11	32.1	16.4	8.4
East Sound (Sand Dollar bed)	10:51	31.9	19.4	8.53
East Sound (Sand Dollar bed)	10:52	31.9	19.5	8.67
East Sound (Sand Dollar bed)	10:54	31.6	20.3	8.71
East Sound (Sand Dollar bed)	10:57	31.7	19.3	8.61
East Sound (Sand Dollar bed)	10:59	31.8	19.1	8.58
East Sound (Sand Dollar bed)	11:00	31.8	20.2	8.64
Mid-way between Sand Dollar Bed & Madrona point (28ft depth)	11:24	31.9	17.7	8.26
Madrona Point (36.7ft depth)	11:27	32.0	17.0	8.37
Rosario	11:43	32.3	15.3	8.30
White Beach	11:53	33.0	12.4	8.05